REGULATION OF SUBCELLULAR MRNA LOCALIZATION IN STRIATAL EFFERENT NEURON SUBPOPULATIONS AND DISRUPTION BY METHAMPHETAMINE

by

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ABSTRACT

The striatum is the key input nucleus of the basal ganglia, which critically mediate normal action selection. Coordinated neuroplasticity within striatonigral and striatopallidal efferent neuron subpopulations of striatum facilitates the maintenance of learned behaviors. In this regard, abnormal striatal plasticity is implicated in compulsive and addictive behaviors. The immediate-early gene, *Arc* (activity-regulated, cytoskeleton-associated), critically mediates such normal neuroplasticity. In normal animals, more striatonigral than striatopallidal neurons contain cytoplasmic *Arc* mRNA, despite equivalent transcriptional activation of the *Arc* gene. Thus, these neuronal populations may differentially regulate *Arc* mRNA posttranscriptional processing and/or cytoplasmic localization and, consequently, utilize different mechanisms of synaptic plasticity. Therefore, these studies determined the subcellular basis for such differential cytoplasmic localization of *Arc* in striatal efferent neuron subpopulations. First, we characterized the striatal expression of eIF4A3, which regulates cytoplasmic *Arc* stability *in vitro* through the process of translation-dependent mRNA decay. Then, to assess whether phenotypic expression differences are unique to *Arc*, we investigated a similarly regulated immediate-early gene, *zif268/egr-1*, which is not subject to cytoplasmic translation-dependent mRNA decay, as well as dendritically localized, but constitutively expressed, *Map2*. Like *Arc*, normal animals had more striatonigral than striatopallidal neurons with *zif268* mRNA in the peri-nuclear cytoplasm, whereas *Map2* expression showed no phenotypic differences. Importantly, striatopallidal neurons had

predominately nuclear *zif268* retention whereas striatonigral neurons had predominately cytoplasmic *zif268* localization. Finally, examining transcriptional activation and cytoplasmic expression of *Arc* in striatopallidal and striatonigral neurons subsequent to partial dopamine denervation induced by methamphetamine (METH) revealed increased basal *Arc* expression, but impaired activity-induced transcription of *Arc* in METH- *vs* saline-pretreated rats. Furthermore, METH-pretreated rats showed blunted cytoplasmic *Arc* localization. Interestingly, cytoplasmic localization of *zif268* was also disrupted by partial dopamine loss, suggesting that dopamine may contribute to cytoplasmic mRNA localization in striatal efferent neurons. The present findings suggest that striatonigral and striatopallidal neurons differentially regulate posttranscriptional processing and/or nuclear export of activity-regulated mRNAs, highlighting a novel, unexplored subcellular difference in striatal efferent neuron subpopulations. These data further suggest that striatal efferent neuron subpopulations may normally utilize different mechanisms of synaptic plasticity to coordinate basal ganglia signaling.

For Matt and Leia – whose countless Utah adventures kept my head clear and ideas big.

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LIST OF SYMBOLS AND ABBREVIATIONS

A2A: Adenosine 2a receptor

AC: Adenylyl Cyclase

AMPA: 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

AMPAR: AMPA-type glutamate receptor

Arc/Arg3.1: activity-regulated, cytoskeleton-associated protein

ASD: autism spectrum disorder

BDNF: brain-derived neurotrophic factor

CaMKIIα: Ca^{2+}/calmodulin-dependent protein kinase

catFISH: cellular analysis of temporal localization by fluorescence *in situ* hybridization

CC: caged control rats

CHX: cycloheximide

CMW: conditioned morphine withdrawal

CPP: conditioned place preference

CREB: cAMP response element binding protein

DA: dopamine

DAT: dopamine transporter

DGlb: lower blade of the dentate gyrus

DGub: upper blade of the dentate gyrus

DIG-UTP: digoxigenin-conjugated UTP

DL: dorsolateral

DM: dorsomedial

DMDA-PatA: *desmethyl,desamino*-Pateamine A

eIF4A3: eukaryotic initiation factor 4A3

EJC: exon-junction complex

ELAV: embryonic lethal, abnormal vision protein

EPSP: excitatory post-synaptic potential

ERK: extracellular signal-regulated kinase

FG: phenylalanine-glycine containing nucleoporins

FISH: fluorescence in situ hybridization

FITC-UTP: fluorescein-UTP

FMRP: Fragile X mental retardation protein

FXS: Fragile X Syndrome

GABA: γ-Aminobutyric acid; IHC, immunohistochemistry

GluA(1-4): AMPA-type glutamate receptor subunit (1-4)

GPe: Globus Pallidus, external segment

GPi: Globus Pallidus, internal segment

HRP: horseradish peroxidase

IEG: immediate-early gene

IP6: inositol hexakisphosate

L-DOPA: L-3,4 dihydroxyphenylalanine

LTD: long-term depression

LTP: long-term potentiation

mACh-R: muscarinic acetylcholine receptors

mRNP: mRNA-protein complex

MANOVA: multivariate analysis of variance

Map2: microtubule-associated protein 2

MAPK: mitogen-activated protein kinase

mEPSC: miniature excitatory post-synaptic current

METH: (±)-methamphetamine hydrochloride

mGluR: metabotropic glutamate receptor

mRNP: mRNA-protein complex

mTOR: mammalian target of rapamycin

NAc: Nucleus Accumbens core

NMDA: *N*-methyl-D-aspartate

NMD: nonsense-mediated mRNA decay

NPC: nuclear pore complex

NXF: nuclear export factor

PBS: phosphate-buffered saline

PBS-T: PBS/0.1% Triton-X

PBS-TB: PBS/0.1% Triton-X/0.1% Bovine Serum Albumin

PKA: protein kinase A

Plk2: polo-like kinase 2

Pol II: RNA polymerase II

ppe: preproenkephalin

ppt: preprotachykinin

PSD: postsynaptic density

RNAi: RNA interference

shRNA: short hairpin RNA

SMD: Staufen-mediated mRNA decay

SNpc: Substantia Nigra pars compacta

SNpr: Substantia Nigra pars reticulate

TDD: translation-dependent decay

TNT: TBS/0.05% Tween-20

TSA: tyramide signal amplification

UTR: untranslated region

zif268: zinc-finger protein 225

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CHAPTER 1

INTRODUCTION

Introduction to the Basal Ganglia

The basal ganglia critically mediate normal behavioral functions by linking cortical activation to the output systems necessary to execute such intended behavior [1]. These output pathways project onto four different brain areas: thalamic nuclei that project to frontal cortical areas mediating action selection and execution of movement; midbrain regions, including the superior colliculus, contributing to eye movements; the pedunculopontine nucleus mediating orientating movements; and finally, hypothalamic structures that regulate autonomic function [2,3]. The major anatomical components of the basal ganglia include the frontal cortex, striatum (caudate and putamen in primates), nucleus accumbens (ventral striatum), external and internal segments of the globus pallidus, subthalamic nucleus, and substantia nigra pars reticulata. Altogether, these nuclei of the basal ganglia coordinate parallel circuits between striatum, thalamus, and cortex to facilitate normal behavioral selection.

The striatum is the key input nucleus of the basal ganglia (Figure 1.1). There are regional divisions within rodent and primate cortex with respect to input onto striatum, with the medial striatum (caudate in primates) primarily receiving excitatory input from prefrontal cortical circuits and the lateral (putamen in primates) receiving excitatory input from the

somatosensory and motor cortices. In addition to the gross neuroanatomical division between medial and lateral striatum, there also exists within the striatum distinct subclasses of GABAergic spiny efferent neurons that can be phenotypically differentiated at the level of their output pathways. Approximately ninety-five percent of neurons in striatum are spiny efferent neurons, and these efferent neurons can be divided into two subpopulations: striatonigral "direct" pathway neurons and striatopallidal "indirect" pathway neurons (Figure 1.1) [4-7]. The striatonigral neurons primarily project to the internal segment of the globus pallidus and substantia nigra pars reticulata, whereas striatopallidal neurons primarily send their axonal projections to the external segment of the globus pallidus [1]. The "direct" pathway neurons selectively provide direct input to the internal segment of the globus pallidus and substantia nigra, and thus reinforce planned movements [5,8,9]. Conversely, the "indirect" pathway neurons selectively project to the external segment of the globus pallidus, which in turn projects to the subthalamic nucleus to modulate cortical activity and terminate movements previously directed by activation of the direct pathway [5,8,9]. This differential and antagonistic output circuitry of striatal spiny efferent neurons contributes to the coordinated regulation of basal ganglia function and thus behavioral selection.

Importantly, striatal efferent neurons also can be phenotypically differentiated at the cell body level by their selective expression of neuropeptides and neurotransmitter receptors. Striatonigral neurons contain preprotachykinin (*ppt*) / substance P, and preprodynorphin / dynorphin [10]. Striatopallidal neurons express preproenkephalin (*ppe*) / met-enkephalin [11]. We will herein utilize the phenotypic neuropeptide expression to differentiate striatal efferent neuron subpopulations. Additionally, these striatal efferent neuron subpopulations also selectively express different receptors, resulting in differential regulation of intracellular signaling cascades and responses to afferents (Figure 1.1). For example, striatonigral neurons

express stimulatory $G\alpha_{s/olf}$ protein-linked D1-type dopamine receptors, which are positively coupled to adenylyl cyclase (AC) [12,13]. Conversely, striatopallidal neurons selectively express inhibitory $G\alpha_{i/o}$ protein-coupled D2-type dopamine receptors [14,15], which inhibit AC activity [16]. Such distinct dopamine receptor expression within specific striatal output neurons leads to differential regulation by dopamine of the activity of AC and thus protein kinase A (PKA) [17], a key second messenger, within these neurons. Additionally, striatopallidal neurons selectively express the $G\alpha_s$-coupled adenosine 2a receptor (A2A) [18]. A2A receptors antagonize D2-dopamine receptor function by reducing the affinity and transduction potential of D2 receptors in response to dopamine [19]. Furthermore, $G_{s/olf}$-coupled A2A receptors activate AC-PKA, and thus oppose the inhibitory effect on AC produced by G_i-coupled D2 receptors [20]. By differentially regulating key cellular receptors and second messengers involved in synaptic plasticity processes, such integrated neurochemical signaling in striatal projection neurons allows for normal, coordinated regulation of their target nuclei (D1-SNpr; D2-GPe; Figure 1.1) and, thus, action selection and behavior [14,21].

Neuronal Synaptic Plasticity

The majority of fast neuronal synaptic transmission within the adult mammalian brain proceeds either through the presynaptic regulation of neurotransmission or through the postsynaptic strengthening or weakening of the efficacy of neuronal connections at synapses via changes in the expression of AMPA-type glutamate receptors (AMPARs). The cycling of AMPARs at the cell surface in synapses contributes to the development of long-term potentiation (LTP) and long-term depression (LTD), forms of synaptic plasticity

implicated in learning and memory [22-24]. Additionally, neuronal homeostatic plasticity regulates longer-duration plastic responses to these subcellular modifications in synaptic transmission and receptor cycling without large-scale modifications of regional synaptic connectivity within neuronal networks [25,26]. Neuronal circuit activity and the history of activity at individual synapses tightly regulate the number, synaptic localization (externalized or internalized), and subunit composition of AMPARs. Thus, neurons can strengthen or weaken their response to excitatory input for durations of minutes to months, and such changes in relative synaptic strength contribute to long-term learning and memory processes within the brain.

One process by which synaptic strength is altered is through LTP, in which there is increased efficiency of synaptic connectivity due to cytoskeletal remodeling, enhanced AMPAR exocytosis, and AMPAR recycling. Much work has identified significant alterations in cytoskeletal architecture during LTP, including the formation of new dendritic spines and the enlargement of existing ones [27-29]. Importantly, fluctuations in F-actin polymerization, shifting between filamentous F- and globular G-actin, result in structural modifications necessary to remodel dendritic spines [30-34], and it is currently believed that a shift in the F-actin/G-actin ratio results in the modifications to dendritic spine structure contributing to LTP [35-37,29]. In dendritic spines, two pools of F-actin exist that directly coordinate synaptic response: one dynamic pool located directly below the spine surface to coordinate AMPAR, NMDAR, and scaffolding protein expression; and a second, less dynamic pool that supports overall spine structure [35,36]. There are increased numbers of new thin, "learning" spines in LTP [38], and late LTP (>3 hours post activity) requires new spine formation [39]. One of the most important and highly enriched postsynaptic molecules contributing to LTP is CaMKII [40,41], which directly contributes to F-actin polymerization through the F-actin

binding domain of the CaMKIIβ subunit [42]. CaMKII activity is essential to structural plasticity, as the targeted blockade of kinase activity disrupts long-term spine enlargement underlying LTP [42,43,29].

Modifications in AMPA receptor surface expression at synapses may contribute to the changes in spine morphology. Furthermore, the surface expression of AMPARs is highly correlated to spine size, with thin, "learning" spines possessing few AMPARs, and large, stable spines having many AMPARs [44]. Localized insertion of recycling endosomes plays an essential role in LTP [45]. Synaptic potentiation drives the Ca^{2+} sensitive motor proteins myosinVa and Vb to deliver cargo vesicles containing AMPARs to the synaptic surface, thus increasing AMPAR surface expression [46,47]. Once inserted at the synaptic surface, there is also lateral diffusion of AMPARs from nonsynaptic dendritic surfaces into the synapse [48-52]. Importantly, GluA1 subunit insertion is essential to the stable enlargement of spines with LTP [53,54]: under basal conditions, GluA1 is not present at synapses, but in response to LTP, GluA1 is driven to the synaptic membrane [52,54]. It is thus clear that AMPA receptor externalization and cytoskeletal remodeling results in increased efficacy of synaptic transmission in LTP, leading to improved ability to depolarize the postsynaptic neuron and thus synaptic strengthening.

In contrast to LTP, cytoskeletal remodeling and receptor trafficking in LTD results in dampening of neuronal responses to excitatory afferents. First, there is both shrinkage and elimination of existing dendritic spines in LTD [55,56]. Additionally, whereas LTP increases the F-/G-actin ratio, LTD shifts the actin equilibrium towards increased G-actin [42]. Third, endocytosis and lysosomal degradation of AMPARs can decrease spine transmission efficiency [24], and AMPARs are known to be subject to such regulation depending on the cellular stimulus [51,57]. For example, glutamate stimulation [58] results in endocytosis of

AMPARs through a clathrin- and dynamin-dependent pathway [59-61] within endocytic zones located lateral to the postsynaptic density (PSD) of dendritic spines [62,51]. Thus, AMPA receptor internalization plays a critical role in LTD.

AMPA receptor internalization is a highly regulated process. For example, in response to glutamate NMDA receptor activation, endocytosed AMPARs are sorted into recycling endosomes in a PKA-dependent manner. Conversely, in response to AMPAR activation, internalized AMPARs are degraded [63,51]. Interestingly, Lee and colleagues further observed that the fate of the internalized receptors is even more highly regulated, with different AMPAR subunits selectively sorted along the endosomal pathway [63]. In response to NMDA receptor stimulation, homomeric GluA2-containing receptors enter the late endosomal pathway and undergo degradation, whereas in response to AMPA receptor activation, the receptors are recycled. GluA1 subunit-containing AMPA receptors, on the other hand, remain in the recycling pathway following NMDA receptor stimulation [63]. These findings suggest that incorporation of the GluA2 subunit defines the endosomal pathway into which AMPARs [63,51,64] will cycle and, thus, AMPAR intracellular fate. Such regulation of AMPA receptors clearly is of high significance, as the coordinated regulation of AMPAR localization on dendritic spines is a critical mediator of synaptic transmission and increased AMPAR internalization is a hallmark feature of LTD.

These immediate modifications of Hebbian forms of synaptic plasticity (LTP and LTD), wherein correlations between pre- and postsynaptic neuronal firing drives postsynaptic changes in the efficacy of synaptic transmission, occur relatively rapidly, and result in localized, ultrastructural modifications to specific synapses in neurons. Such forms of plasticity are dependent on neuronal firing patterns and progressively modify neuronal connectivity to make associative changes in response to such stimuli, ultimately resulting in

neuronal destabilization due to the increased or decreased ease with which a postsynaptic neuron can subsequently be depolarized by the presynaptic input [65]. To counterbalance these rapid, destabilizing changes, neurons also undergo homeostatic plasticity, which stabilizes their own activity, as well as circuit-level activity [24]. Homeostatic plasticity is thus a more global mechanism that neurons and neuronal circuits utilize to maintain a specific level of activity or parameter "set point" [26]. Should signals or activity deviate from this set point, the neurons/system respond to return activity in the necessary direction. Thus, these more universal compensatory mechanisms in a neuron can be activated to maintain normal activity while leaving synapse-specific changes in strength relatively maintained.

Homeostatic plasticity allows neurons to dynamically respond to stimulation specifically through the scaling up or scaling down of synaptic transmission to calibrate neuronal excitability. One the one hand, increased activity, over time, leads to a global decrease in excitability and thus, normalization of firing. For example, hippocampal and cortical neuron cultures induced to fire repeatedly over many hours eventually return firing rates to basal levels [66,67]. Mechanistically, extraneous synaptic activity results in enhanced intracellular calcium, gene transcription, CaMKIV activation, and polo-like kinase 2 (Plk2)/Cdk-5 activation [68,69]. Plk2 activation phosphorylates factors which regulate the calcium-impermeable, slowly deactivating GluA2 subunits [68,70], resulting in a reduction in activity-induced AMPAR accumulation in synapses [69], and thus, a scaling down of synaptic transmission. An additional scaling down pathway has recently been demonstrated to depend on activation of the immediate-early gene *Homer1a*, resulting in reduced tyrosine phosphorylation of GluA2 [71,26]. On the other hand, decreases in neuronal activity are associated with an overall increase in neuronal excitability, termed "scaling up," which also is secondary to alterations in AMPA receptor expression and cell-surface insertion. For

example, blocking postsynaptic neuron firing leads to decreases in somatic calcium, CaMKIV activation, and gene transcription [72]. Also, the AMPAR binding protein, PICK1, specifically regulates the intracellular pool of receptors, selectively binds to GluA2 receptors [73], and is essential to scaling up, but not scaling down [26]. These intracellular scaling responses then result in enhanced AMPAR externalization and increased amplitude of miniature excitatory postsynaptic currents (mEPSCs). Such cell-autonomous changes in synaptic scaling are exquisitely different from LTP and LTD and operate over a longer duration within a wider spatial range in a neuronal network, employing distinct cellular trafficking and molecular events to regulate AMPARs within a neuron [26], thus making homeostatic plasticity an additional means by which neurons and neuronal circuits respond to stimuli.

Striatal Synaptic Plasticity

The dorsal striatum is involved in reward processing, goal-directed behaviors, and habit formation [74,75,1,9]. Synaptic plasticity within this brain region is thus critical to the acquisition and maintenance of such learned behaviors [76,77]. Abnormal signaling and plasticity in the basal ganglia is implicated in compulsive and addictive behaviors, as well as behavioral sequelae of neurodegenerative disorders, such as Parkinson's and Huntington's disease [78-82], including L-DOPA-induced dyskinesias [83-85]. Thus, understanding the mechanisms underlying LTP, LTD, and homeostatic plasticity in striatal efferent neurons will be critical for advancing our ability to prevent or manage neurological and neuropsychiatric consequences of basal ganglia dysfunction.

Much of our understanding of synaptic plasticity processes in general has been developed based on studies of hippocampal and cortical systems (see for example [86]), as

reviewed above. Unfortunately, the neuroanatomical heterogeneity of striatal projection neurons has slowed our understanding of plasticity-related cellular processes in the striatum *in vivo*. Importantly in this regard, recent work by Baucum and colleagues highlighted selective, but significant, differences between the hippocampus and striatum at the level of the assembly of synaptic and extrasynaptic signaling complexes essential to neuroplasticity processes [87]. Thus, it is essential to further examine the specific cellular and molecular processes mediating striatal neuroplasticity in identified striatal efferent neuron populations so as to better understand both normal, as well as aberrant, functions within this key nucleus of the basal ganglia. By fully understanding normal mechanisms underlying neuronal synaptic plasticity processes within the striatum, novel methodologies and therapeutic targets can be identified to improve treatment strategies for disorders of the basal ganglia, including drug abuse and addiction, and movement and compulsive disorders.

Striatal neuron plasticity is regulated by glutamate derived from cortical and thalamic afferents, dopamine derived from nigral afferents, and calcium. Glutamate input alone seems to result in the development of LTP of synaptic transmission [88,89], whereas glutamate input coincident with dopamine input onto striatal spiny efferent neurons can induce LTD of synaptic transmission [88,89]. Additionally, removing the magnesium block from the NMDA receptor results in calcium influx that can induce LTP in the absence of dopamine [90]. Furthermore, in response to presynaptic stimulation following postsynaptic spikes (10 ms), spiny efferent neurons show specific differences in response to stimulation; cultured D2 receptor-containing spiny efferent neurons (presumed striatopallidal) show LTD, whereas D1 receptor-containing spiny efferent neurons (presumed striatonigral) show no change in excitatory postsynaptic potential (EPSP) amplitude, indicating no induction of LTD or LTP [91]. Furthermore, Shen and colleagues demonstrated that selectively blocking D1 receptors

during this paradigm of pairing postsynaptic spiking followed by presynaptic activity resulted in robust LTD in D1 receptor-containing spiny efferent neurons, thereby demonstrating that DA is required for LTP induction in D1 receptor-containing neurons (presumed striatonigral) [91]. These studies demonstrate that spiny efferent neurons show a differential response to pre- and postsynaptic stimulation pairings, indicating that DA is required to induce bidirectional, timing-dependent plasticity at glutamatergic synapses in medium spiny neuron cultures and that such plasticity is DA receptor content-dependent [91]. As normal, tonic firing of DA neurons results in sufficient levels of DA to stimulate D2-, but not D1-, containing DA receptors in medium spiny neurons [92,93], the absence of behaviorally-relevant stimuli (i.e., low extracellular DA concentration) would normally result in LTP in striatopallidal neurons, but should only induce LTD in striatonigral neurons [91]. On the other hand, phasic DA release in response to behaviorally-relevant stimulation will sufficiently activate D1-containing DA receptors to induce LTP in striatonigral neurons [93,92,91]. Thus, under periods of fluctuations in behavioral activation, DA can differentially modulate the plasticity response within striatonigral and striatopallidal neurons. Such data highlight the myriad ways in which synaptic efficacy in striatal medium spiny neurons can be modified through both glutamate and dopamine signaling, as well as providing an intriguing explanation for divergent roles that medium spiny neurons play in regulating behavior.

Striatal neurons rapidly respond to excitatory input due to their expression of AMPA-type glutamate receptors on the cell surface [23,94]. Heteromeric AMPA-type glutamate receptors are composed of subunits GluA1-4, with GluA1 and GluA2 subunits predominating within striatum [95-98]. Importantly, subunit composition critically determines the biophysical properties of AMPA receptors [99-102]. GluA2 subunit-containing AMPA receptors are Ca2+ impermeable, show smaller conductance, and

desensitize and deactivate more slowly than do GluA1 subunit-containing receptors [103-106]. Furthermore, the presence or absence of GluA2 subunits contributes to changes in synaptic strength [107,108], as detailed above. AMPA receptor functional properties also are acutely responsive to phosphorylation of the GluA1 subunit at two separate sites [102]. Phosphorylation by PKA at Ser845 increases the open probability of the channel and drives synaptic membrane insertion [109-111]. For example, within striatal spiny efferent neurons, PKA-dependent phosphorylation of GluA1 at Ser845 results in enhanced AMPA receptor surface expression [112,113]. Phosphorylation of GluA1 at Ser831 [114,115] by protein kinase C and calcium/calmodulin-dependent protein kinase II results in increased AMPA channel conductance [116]. As previously detailed, increases in AMPAR surface expression and function result in LTP, whereas removal of synaptic AMPARs results in LTD [24]. Thus, the subunit composition and subunit phosphorylation state of AMPA receptors can critically regulate striatal synaptic plasticity.

Within the striatum, AMPAR subunit distribution likely contributes to factors underlying differences in synaptic plasticity in striatal efferent neuron populations. Although AMPARs can be comprised of multiple subunits, immunohistochemical staining and coimmunoprecipitation for GluA1, GluA2/3, and GluA4 subunits within dorsal striatum demonstrate that GluA1 and GluA2/3 subunits predominately colocalize with striatal projection neurons [117-119,98]. However, ultrastructural imaging of the spines of striatal efferent neuron subtypes suggests that there are distinct differences in subunit expression, with striatonigral dendritic spines more commonly containing GluA2 and/or GluA3 subunits, whereas striatopallidal dendritic spines more commonly contain GluA1 subunits [120]. As described previously, subunit composition determines routes of AMPAR trafficking, with GluA1 determining AMPAR fate during exocytosis, and GluA2 regulating

endocytosis and postendocytotic endosomal sorting [24]. In the absence of GluA2 surface expression, maintenance of LTP is abolished, and there is Ca^{2+}-dependent intracellular retention of GluA2 in endosomal compartments during LTD [24]. As GluA2 subunits predominately control AMPAR recycling and endocytotic sorting in response to changes in synaptic transmission efficiency, such divergent distribution of GluA2 expression on the spines of striatal efferent neurons provides another basis for neuroplasticity processes within striatal efferent neurons to be differentially regulated.

Neuromodulators critical to striatal neuron function may differentially contribute to regulation of AMPA receptor trafficking and surface expression in the subtypes of striatal projection neurons. Specifically, AMPA GluA1 subunits are phosphorylated at Ser845 by PKA downstream of D1-type dopamine receptor activation [113,121]. Furthermore, using the D1-receptor agonist SKF 81297, Mangiavacchi and Wolf demonstrated that GluA1 subunit surface expression is enhanced, in a PKA-dependent manner, in ventral striatum following D1 receptor activation [112]. Given that D1-type dopamine receptors are selectively expressed in striatonigral neurons [14,122], it is likely that such D1 receptor activation contributes to enhanced surface expression of AMPA receptors in striatonigral efferent neurons [113]. Conversely, activation of D2-type dopamine receptors, predominately expressed in striatopallidal neurons [14], inhibits GluA1 subunit phosphorylation at Ser845, resulting in AMPA receptor endocytosis [123]. Thus, differences in AMPA receptor subunit composition in specific striatal efferent neuron populations, as well as differential regulation of AMPA receptor subunit surface expression by dopamine signaling within those neuronal populations may contribute to differential forms of synaptic plasticity processes, including LTP [94,23], LTD [23], and homeostatic synaptic scaling [124,125] within striatal efferent neuron subpopulations. Further understanding the

processes and molecules that thus regulate AMPA receptor surface expression within striatal efferent neurons will afford improved direction in developing therapeutic interventions for basal ganglia-mediated pathologies.

Arc and Neuroplasticity

The immediate-early gene *Arc* (activity regulated, cytoskeleton-associated protein) is an important molecule that contributes to normal AMPA receptor surface expression and, thus, neuroplasticity. *Arc* mRNA expression and subcellular distribution is activity regulated [126,127], and the mRNA is localized to dendritic spines in response to neuronal activation where it undergoes localized protein translation [128]. Importantly, knockdown of *Arc* impairs both hippocampal- [129,130] and basal ganglia-mediated learning and memory processes [131,132], thereby highlighting the critical role *Arc* plays in mediating normal synaptic plasticity processes, including those in the striatum.

As a synaptic protein, Arc likely makes several contributions to processes involved in synaptic remodeling [133]. First, Arc appears to play a role in LTD. For example, Arc protein localizes to the PSD of excitatory synapses [126,134], where it interacts with endophilin 2/3 and dynamin to form a trafficking endosome to regulate endocytosis of AMPA receptors [135-137]. Consistent with this role mediating endocytosis, overexpression of Arc protein reduces the amplitude of synaptic currents mediated by AMPA receptors [136]. Furthermore, Arc regulation of AMPA-receptor endocytosis appears to be different for AMPA receptors comprised of different subunits, although the specifics of such regulation are currently less clear. On the one hand, Arc protein has been reported to result in the global removal of GluA2/3 subunit-containing AMPA receptors in neurons in hippocampal slice preparations [136,137]. Conversely, Peebles and colleagues recently

demonstrated that Arc protein specifically plays a role in internalizing GluA1 subunit-, but not GluA2/3 subunit-, containing AMPA receptors in thin "learning" spines [44] in cultured hippocampal neurons, suggesting that Arc protein may directly facilitate modifications in spine morphology through the selective regulation of GluA1 internalization. Furthermore, Waung and colleagues recently demonstrated that Arc critically regulates GluA1 endocytosis, as induction of mGluR-LTD led to rapid dendritic Arc protein synthesis, and knockdown of Arc protein specifically disrupted the activity-dependent decreases in GluA1 surface expression and disrupted the onset of LTD [138]. Finally, Okuno and colleagues have recently demonstrated that Arc specifically interacts with CaMKIIβ to create an inverse synaptic tag that senses inactive synapses, thereby specifically increasing GluA1-containing AMPAR endocytosis [139]. Thus, *Arc* appears to play a crucial role in regulating the development of LTD.

Arc also appears to play a critical role in the regulation of plasticity processes in dendritic spines contributing to LTP. For example, Arc regulates F-actin stabilization essential to LTP [129,30,31]. Second, in studies using neuronal cultures in which the endocytic functions of Arc are disrupted, the normal activity-induced increase in thin "learning" spine formation is disrupted [44]. Additionally, *Arc* expression *in vitro* increases spine density and knockdown of *Arc* mRNA decreases spine density, thereby impairing LTP [44]. *Arc* mRNA expression is also correlated to dendritic spine density in the amygdala in a rodent model of alcoholism [140], providing further translational relevance to the role of *Arc* mRNA in mediating neuroplasticity processes. In light of these findings, it is clear that *Arc* critically regulates both normal and aberrant plasticity processes, particularly via regulation of AMPA receptor surface expression.

Recent *in vivo* studies using site-specific *Arc* antisense oligonucleotide infusions (*Arc* AS) or RNA-interference (i.e., shRNA/RNAi) to disrupt *Arc* expression in specific brain regions have demonstrated the essential role that *Arc* plays in mediating normal long-term memory processes [130,141]. One of the first studies to disrupt *Arc* signaling within the basal ganglia was performed by Ploski and colleagues [142] who were examining memory consolidation in the lateral amygdala following Pavlovian fear conditioning. Infusions of *Arc* AS into the lateral amygdala disrupted long-term (24 hr), but not short-term (3 hr), fear memory [142]. Likewise, place aversion induced by conditioned morphine withdrawal (CMW) is blocked following lentiviral vector-mediated delivery of *Arc* shRNA to the basolateral amygdala. Interestingly, in relation to the discussion of AMPA regulation detailed above, such disruption of *Arc* signaling results in significant elevations in surface AMPA GluA1 and GluA2 surface expression relative to control animals following CMW [31]. Thus, knockdown of *Arc* within the amygdala impairs retention of learned behaviors (i.e., fear conditioning and conditioned place aversion) and this impairment is associated with disrupted AMPAR endocytosis.

In the basal ganglia, disruption of *Arc* also impairs long-term consolidation of basal ganglia-mediated learning. For example, infusion of *Arc* AS into the nucleus accumbens (NAc) core and shell of rats significantly impaired morphine-induced conditioned place preference (CPP) [143]. Additionally, Hearing and colleagues have shown that *Arc* AS infused into the dorsal striata of rats impairs extinction of context-induced cocaine-seeking and also significantly reduced GluA1 subunit- and increased GluA2 subunit-surface expression in striatum [132]. Finally, Pastuzyn and colleagues have also recently demonstrated an essential role for *Arc* in contributing to dorsal striatum-mediated motor response reversal learning, as rats receiving *Arc* AS infusions failed to retain previously

acquired behavioral performance on a response reversal T-maze task relative to rats infused with PBS or a scrambled oligonucleotide [131]. Thus, overwhelming evidence illustrates a crucial role for Arc in facilitating synaptic plasticity necessary for learning and memory processes in the brain in general, and in the basal ganglia in particular. As such, *Arc* represents a pivotal tool through which to understand both normal and aberrant synaptic plasticity in striatal efferent neuron subpopulations of the basal ganglia.

In addition to the phenotypic expression of neuropeptides and receptors within striatonigral and striatopallidal neurons delineated previously, we have repeatedly demonstrated that more striatonigral neurons contain *Arc* mRNA in the cytoplasm than striatopallidal neurons, despite similar numbers of these neurons showing transcriptional activation of the *Arc* gene [144,145]. Thus, striatal efferent neuron subpopulations somehow differentially regulate *Arc* mRNA nuclear posttranscriptional processing and/or cytoplasmic stability and protein translation. Given the critical role of Arc in regulating AMPAR endocytosis and synaptic plasticity processes, this difference in cytoplasmic *Arc* mRNA availability may lead to differences in AMPAR surface expression and thus synaptic plasticity processes within striatonigral and striatopallidal neurons. In theory, such differences may contribute to striatal neuron subpopulation-specific induction of LTP in response to DA input [91], as detailed above. As normal behavior selection depends on appropriate, coordinated activity between striatonigral and striatopallidal efferent neurons [146], the difference in cytoplasmic *Arc* mRNA availability is intriguing and provides a means to further expand our understanding of both normal and aberrant synaptic plasticity processes within the basal ganglia.

While it is now well established that more striatonigral than striatopallidal neurons have *Arc* mRNA in the cytoplasm [145,144,147], what is less clear is whether this difference

reflects a feature unique to *Arc* mRNA *vs.* global differences in striatal neuron posttranscriptional processing of mRNAs. Although much work into factors and processes contributing to general neuronal mRNA regulation has been conducted both *in vivo* and *in vitro*, how these factors specifically alter mRNA subcellular distribution within striatal efferent neuron subtypes *in vivo*, and thus basal ganglia signaling in general, remains undefined. The remaining section of this introduction will thus be dedicated to exploring the vast, dynamic regulatory mechanisms contributing to both constitutive and activity-regulated neuronal mRNA signaling. Discussions of the potential impact of such subcellular regulation on the previously observed differences in *Arc* mRNA expression will be discussed where relevant, with other exemplary synaptic plasticity-associated genes included in pertinent sections, as well.

Neuronal RNA Regulation and Implications for Striatal Signaling

The polarized nature of neuronal cells requires tight regulation of cellular responses being transmitted from the nucleus to activated synapses. Neurons achieve diverse responses to myriad synaptic inputs by tightly controlling gene expression on multiple levels, including: transcriptional activation, posttranscriptional processing, cytoplasmic mRNA stabilization, micro-RNA regulation of mRNAs, localized mRNA transport, and localized protein translation in dendrites. In the following sections, we will explore these myriad regulatory pathways and how such regulation may contribute to factors regulating synaptic plasticity, and thus learning and memory processes, within the striatum and basal ganglia. Because of the extensive research on the posttranscriptional processing and trafficking of *Arc*, as well as our own work into its expression and regulation within the basal ganglia, we will focus on this gene. However, where relevant, discussion of other candidate genes that contribute to

synaptic plasticity processing and that demonstrate prototypical characteristics pertinent to a particular section will be included.

Transcriptional Control and Nuclear Packaging

Neurons rapidly respond to synaptic stimuli through a class of rapidly transcribed immediate-early genes (IEGs) that encode transcription factors and effector proteins involved in cellular response and remodeling processes. Such IEGs are unique in that they require no *de novo* protein synthesis to induce their transcription but can, in turn, activate the expression of downstream cellular targets [148]. It has been estimated that some 30-40 neuronal genes are IEGs, with approximately 10-15 encoding neuronal transcription factors and the remaining genes encoding effector IEGs [149,150]. This diversity of IEGs differs from the constitutively expressed transcription factors, such as Elk-1 and cAMP-response element binding protein (CREB), in that they are activated on the order of minutes after stimulation. Prototypical inducible transcription factor IEGs include *zif268/egr-1* and *c-Fos*, whereas effector IEGs directly involved in synaptic scaling and plasticity processes include *Arc* (as described in detail above) and *Homer1a* [149]. Importantly, while synaptic activation induces IEG transcription, the early phases of LTP are dependent on second messengers and kinase activity. It is only the later phases of LTP that depend on transcription and translation, with IEGs playing a major role in mediating such long-term synaptic modifications [148,151]. Thus, rapid activation of cellular signaling pathways inducing IEG mRNA transcription gives neurons the ability to quickly respond to changes in synaptic stimulation.

The rapid cellular response to changes in intracellular Ca^{2+} concentration following synaptic stimulation can activate or suppress transcriptional activity of IEGs essential to

LTP and LTD. Synaptic activity rapidly induces both *zif268* and *Arc* mRNA transcription via activation of NMDA-type glutamate receptors and the extracellular signal-regulated kinase (ERK) signaling pathway [133,128,126,127,152]. Both *zif268* and *Arc* contain serum response elements (SREs) within their promoters [153-157], tightly coordinating the rapid induction of these genes in response to cellular stimuli. In the case of *zif268*, SREs are the main activation site necessary for mRNA induction [157], while other regulatory factors acting at individual binding sites, such as CREB [156,158] or Activator Protein 1 (AP-1) [158,157], play more modulatory roles in the regulation of *zif268* transcription [155,148]. Furthermore, neurons can rapidly induce transcription of some IEGs, in particular *zif268* and *Arc,* through the poised stalling of RNA Polymerase II (Pol II) just downstream of the transcription start site [159]. Under basal conditions and prior to any second messenger activation, Pol II transcribes approximately 20-25 nucleotides of these rapid IEGs, then stalls on the IEG promoter and sits primed until an action potential triggers changes in intracellular calcium concentration[160], leading to the dissociation of Negative Elongation Factor (NELF) from Pol II, thereby allowing Pol II transcription to continue. In the absence of such Pol II stalling, rapid induction of *zif268* and *Arc* mRNA is abolished [159]. Thus, neurons tightly control the transcriptional activation of IEGs through the coordinated response to excitatory input and the poised stalling of Pol II on the IEG promoter, allowing for rapid neuronal response to synaptic stimulation.

Stimulation-dependent regulation of transcriptional activation is, however, just the first stage within the nucleus by which neurons tightly sculpt responses to synaptic activation. Following this rapid transcription, pre-mRNAs can undergo complex posttranscriptional splicing via the spliceosome to regulate the information being conveyed from DNA to synapses, with spliced mRNAs ultimately possessing higher translational

capacity than nonspliced mRNAs [161]. The Homer family of scaffolding proteins, for example, signals through different *Homer* transcripts depending on the posttranscriptional splicing [162]. *Arc* is also subject to pre-mRNA splicing, whereby introns within the 3' untranslated region (UTR) are removed and exon-exon junction protein complexes (EJC) loaded upstream of the ligation site (Figure 1.2) [163-166]. The EJC is anchored to *Arc* by the DEAD-Box RNA helicase, eIF4A3 [167,168,164]. Importantly, the EJC is involved in splicing events for multiple neuronal mRNAs [169,170,166], mediates normal mRNA quality control through nonsense-mediated mRNA decay (NMD) [171-173], and travels with spliced mRNAs until they are degraded or translated by the ribosome [174-176]. However, although other neuronal mRNAs, such as *Map2* and *CaMKII*α, precipitate with eIF4A3, they do so to a much lesser extent than does *Arc* [166], suggesting a role of the EJC in selectively regulating certain neuronal mRNAs. Thus, addition of the EJC is a critical posttranscriptional regulatory step for mature mRNAs that proceeds within the nucleus [177]. Furthermore, such posttranscriptional addition of the EJC has recently been demonstrated to mediate synaptic plasticity processes within cultured hippocampal neurons [166], thereby further implicating posttranscriptional splicing and processing as a critical step underlying neuronal signaling and plasticity.

Following nuclear splicing of the pre-mRNA, the mature mRNA is packaged and prepared to exit the nucleus. In addition to the core EJC proteins added within the nucleus, the nuclear protein UAP56, a core component of the TREX protein complex essential to mRNA export [178], transiently associates with EJC proteins to couple spliceosome assembly to nuclear export of mRNA-protein complexes (mRNPs) [179-182]. UAP56 is essential to mRNA packaging and export due to its key role in recruitment of the export factor, Aly/REF, onto spliced mRNAs (Figure 1.2) [183,163,184]. Aly/REF then serves as

the adaptor protein necessary to recruit the principal mRNA Nuclear eXport Factor (NXF) TAP/NXF1 onto mRNAs [185-188], creating an export-competent mRNP. This export-competent mRNP, containing the EJC [189,190,173], Aly/REF, and TAP/NXF1, then travels via Brownian motion to the nuclear pore complex (NPC) for nuclear export [191,192]. Thus, in addition to tight transcriptional control, multiple levels of splicing and packaging occur within the nucleus prior to export of the mRNP. Such discrete intranuclear regulation contributes to the added complexity of gene expression within activated neurons.

A comprehensive understanding of the expression of these nuclear packaging and export factors within the adult mammalian brain is only just beginning to be achieved now that these components and pathways have undergone more and more scrutiny in model organisms such as *Saccharomyces cerevisiae* and *Drosophila melanogaster*. Interestingly, in mice, the NXF family of proteins, of which Tap/NXF1 is the principal export factor, is encoded by at least four genes (Tap/NXF1, NXF2, NXF3, and NXF7). In mouse hippocampal neurons, NXF2 also acts as an mRNP exporter and specifically interacts with kinesin KIF17 [193]. This is of intriguing relevance to striatal synaptic plasticity, as KIF17 transports NR2B subunit-containing NMDA receptors within dendrites [194], and overexpression of KIF17 enhances spatial and working memory in transgenic mice [195]. Moreover, NR2A and NR2B subunits predominate within medium spiny projection neurons striatum [196,197]. As described above, NMDA receptors are critical to postsynaptic signaling necessary to induce *Arc* and *zif268* mRNA transcription [155,135], thus NXF2 could potentially contribute to striatal neuroplasticity. Moreover, NXF2, but not NXF1, specifically interacts with Fragile X mental retardation protein (FMRP) [198], and NXF2 and FMRP together destabilize the *NXF1* mRNA *in vivo [199]*, thereby serving as a posttranscriptional regulation complex of the principal mRNA export factor, NXF1. With FMRP playing a known role in *Arc*-

dependent neuroplasticity processes [200,201] and dysfunction of this protein contributing to neurological disorders [202-205], understanding the expression of the export factors NXF1 and NXF2 *in vivo* is of critical importance to advancing our understanding of striatal neuroplasticity processes. How these factors and protein families are expressed in neurons of the mammalian brain in general, and within striatal efferent neurons in particular, will likely afford a more comprehensive understanding of the means by which neurons communicate in response to synaptic activation.

Nuclear Export of mRNPs

Distribution of mRNPs from the nucleus to the cytoplasm via export through the NPC is another "speed-bump" on the road to nuclear communication with synapses. As delineated above, export competent mRNPs, with TAP/NXF1 bound, exit the nucleus through the NPC (Figure 1.2). TAP/NXF1 interaction with FG(phenylalanine-glycine)-containing nucleoporins facilitates export of the mRNP through the NPC [206-209]. Most evidence suggests that export through the NPC is a relatively fast process in metazoans [210,211]. However, cytoplasmic retention of exported mRNPs requires the DEAD-box RNA helicase DDX19 (in yeast, Dbp5), which catalyzes the removal of TAP/NXF1, thereby remodeling the mRNP and preventing it from returning to the nucleus [212,213]. DDX19 is recruited to the NPC by the nucleoporin NUP214 (yeast, Nup159) and there binds to the NPC protein GLE1 (yeast, Gle1) [191]. Importantly, DDX19 requires local activation by GLE1 and its co-factor inositol hexakisphosphate (IP6) to stimulate ATPase activity and thus RNA binding to mediate cytoplasmic mRNP remodeling [214,215]. DDX19:GLE1 interactions are the rate-limiting step in nuclear export of mRNPs, as mutations in GLE1 interactions with DDX19 result in nuclear accumulation of mature,

polyadenylated mRNAs, whereas alterations in the NUP214:DDX19 interaction have no effect on nuclear export [215]. Further, IP6 is critical to the stabilization of DDX19:GLE1 interactions *in vivo*, and is thus critical to mRNA export [214]. In the absence of IP6 or GLE1, DDX19 activity cannot proceed, leading to nuclear accumulation of mRNPs [216,217]. Thus, while export through the NPC is relatively fast, mRNP retention within the cytoplasm appears to be critically modulated by the activity of DDX19.

Interestingly, in the context of hippocampal neuroplasticity, IP6 can activate PKA [218], specifically participates in AMPA, but not NMDA, receptor localization [219], and is critical to L-type Ca^{2+} channel activity [218]. Thus, it is plausible that a similar role for IP6 would exist within striatal efferent neurons, likely contributing to striatal neuroplasticity processes, as well. While the interactions of DDX19 with RNA and GLE1:IP6 are now quite well understood in model systems [220], what remains to be further defined is how cytoplasmic mRNP remodeling proceeds in neurons, and how cellular differences in such remodeling processes might contribute to phenotypic differences in mRNA expression and function, such as the differences observed for *Arc* mRNA in striatal efferent neurons [145,144].

Cytoplasmic mRNA Trafficking, Localized Protein Synthesis, and mRNA Stability

Within neurons, *de novo* protein translation is critical to the induction, consolidation, and maintenance of late-LTP [221-224]. Neurons can tightly regulate the information being conveyed at synapses through delivery of mRNP granules for local protein synthesis. Importantly, mRNPs are dendritically localized by kinesin motor proteins along microtubules to synapses [225,226], and dendritic spines contain the polyribosomes and machinery necessary for such local protein synthesis [227]. Furthermore, polyribosomes

translocate from dendritic shafts to dendritic spines containing enlarged heads in response to LTP-inducing stimulation [228-230]. While only a select fraction of mRNAs are IEGs that transcriptionally activate in response to synaptic stimulation [150], many mRNAs undergo activity-dependent synaptic localization and protein synthesis [231], including *Arc* [232], *COX-2* [233,234], *CaMKIIα* [235,236], and *Map2* [237]. Localized mRNAs such as *Arc* [238], *CaMKIIα* [239,240], and *Map2* [241], contain *cis*-acting elements within the 3'UTR that signal dendritic targeting. These *cis*-acting elements are critical to normal neuronal function. For example, in mice lacking the *CaMKIIα cis*-acting element, *CaMKIIα* mRNA is restricted to the cell body, and the mice show a significant reduction in late-phase LTP and memory impairments [242]. These *cis*-acting elements interact with transcript-specific, *trans*-acting RNA-binding proteins to target the mRNP to synapses [243,244]. Such *trans*-acting proteins, including FMRPs (FXR1 and FXR2, FMR1 [245]) and Staufen [246], play a critical role in delivery of RNA granules [225] and thus directly contribute to neuroplasticity processes (Figure 1.3) [247,248]. Future research into the expression and regulation of such *trans*-acting factors within striatal efferent neuron subpopulations could provide valuable understanding of the processes by which spiny efferent neurons respond during periods of activation, as well as how aberrant plasticity, such as in the context of drug abuse and addiction, may alter such signaling [249].

Once delivered to the neuronal cytoplasm, mRNAs can be trafficked to and undergo rapid protein synthesis at the appropriate synapse or remain translationally dormant until recruited to the appropriate synapses. For example, newly synthesized Arc protein is detected within the same dendritic regions as newly synthesized *Arc* mRNA [128], demonstrating that *Arc* mRNA is rapidly trafficked and translated in response to synaptic

activation. Conversely, both *CaMKIIα* and *Map2* mRNAs are constitutively expressed in neurons [237,250,235,251]. *CaMKIIα* mRNA is stored in pools of translationally dormant mRNA within hippocampal neurons until there is activity-dependent delivery of pre-existing mRNAs to synaptic sites [236]. In as little as 5 minutes after high-frequency stimulation (HFS), *CaMKIIα* protein is detected >100 um from the neuronal cell body [235]. As for constitutively expressed *Map2* [237], HFS results in a two-stage alteration in dendritic immunostaining [251]. Brief HFS trains (5 minutes) lead to decreased immunostaining in the dendritic region of the dentate gyrus receiving synaptic stimulation, while longer HFS trains (2 hr) result in increased Map2 immunostaining on each side of the activated lamina [251], demonstrating time-dependent control of Map2 protein degradation (brief HFS) and localized mRNA delivery and protein synthesis (long HFS) within activated neurons. Whether behavioral activation induces such changes in CaMKIIα and Map2 protein synthesis in striatum has yet to be described, but preliminary *in situ* hybridization histochemistry studies suggest that striatal neurons also show activity-dependent trafficking of both *CaMKIIα* and *Map2* mRNAs away from the cell soma in response to behavioral activation [*CaMKIIα*, Barker-Haliski and Keefe, unpublished; *Map2,* Chapter 3]. Thus, localized, rapid protein synthesis occurs in response to synaptic activation and is thought to critically mediate normal neuronal synaptic plasticity processes.

As described in detail above, late-LTP and LTD require *de novo* protein synthesis of localized mRNAs. Ribosomes, translation factors (such as the translation initiation complex eIF4F and its repressor protein 4E-BP), and plasticity-associated mRNAs are present in dendrites and dendritic spines, as well as within the cell body, allowing neurons to tightly regulate long-lasting synaptic plasticity even without activation of nuclear transcription

[252,227,253]. Such protein translation is dynamically regulated by multiple cell signaling events, including activation of mammalian target of rapamycin (mTOR)-mediated phosphorylation of 4E-BP and ribosomal S6 kinase (S6K) [254,253], thereby releasing inhibition of the eIF4F cap-binding complex and allowing this complex to form on target polyadenylated mRNAs, resulting in translation initiation [255,228]. Importantly, LTP-inducing stimulation activates mTOR, and thus phosphorylation of 4E-BP2 (the predominate 4E-BP protein) in the mouse brain, resulting in elevated eIF4F complex formation [256,253,257]. Interestingly, 4E-BP knockout mice show a decreased threshold to induce late-LTP, likely as a result of the increased eIF4F complex formation and translation initiation [257]. Such mice also show deficits in hippocampal-dependent spatial learning and memory processes [257]. An additional translational regulatory pathway is the through the RNA-binding protein, FMRP [245], with a role in specifically regulating *Arc* mRNA translation and thus LTD recently coming to light [200,258]. FMRP functions as a translational repressor for a number of synaptic plasticity-associated mRNAs, including *CaMKII*α and *Map1b* [201,138,253], that are essential to endocytosis of AMPARs and LTD. Thus, translational control of synaptic plasticity-associated proteins at activated synapses contributes to the activity-dependent scaling of neuronal response by LTP and LTD.

In addition to being translated into protein, once in the cytoplasm, mRNAs in mRNPs can also be subject to cytoplasmic regulatory events en route to or at their destination. One such mechanism contributing to normal mRNA quality control is nonsense mediated mRNA decay (NMD; Figure 1.2) mediated by the EJC [259,171]. Generally during the first round of protein translation, the ribosome removes EJCs as it translates through exon-exon boundaries [175,260]. However, if the mRNA exists in such a way that an exon-exon junction falls outside the reading frame and, thus, the EJC persists on the mRNA after

the first round of translation, that mRNA is targeted for NMD [261], a process mediated by EJC interaction with UPF1 [262-264]. In the classical pathway of NMD [259], UPF1 phosphorylation initiates recruitment of specific mRNA decay factors (Figure 1.3 [265,266,262]). Specifically, Dcp1 cleaves the 5'-end cap to allow for the 5'-to-3' exonuclease Xrn-1 and the 3'-to-5' exonuclease Rrp4 to degrade mRNA transcripts, thereby tightly regulating cytoplasmic mRNA stability and mRNA abundance [259,171,261]. What is most interesting, however, is that a select fraction of mRNAs are subject to the NMD-like process of translation dependent decay (TDD) mediated by the EJC [166], because these transcripts contain EJC proteins within the 3'UTR that are not removed during the pioneer round of translation [164,166]. In particular, *Arc* mRNA is a natural target for TDD due to the two EJCs within the 3'UTR (Figure 1.2), leading to tight translational control of this mRNA and, thus, Arc protein synthesis (Figure 1.3) [166,267]. Given that eIF4A3 is critical to the EJC, it may act as a brake on cytoplasmic *Arc* mRNA availability. Indeed, Giorgi and colleagues [166] demonstrated the role of eIF4A3 in TDD of *Arc* mRNA, as knockdown of eIF4A3 resulted in a 2-fold increase in *Arc* mRNA and a 4-fold increase in Arc protein levels in both hippocampal somata and dendrites [166]. Knockdown of eIF4A3 also resulted in increased miniature excitatory postsynaptic current (mEPSC) amplitude and AMPA receptor levels at hippocampal synapses [166]. Thus, eIF4A3 offers an intriguing means to potentially modulate synaptic plasticity processes in striatal efferent neurons due to its core role in EJC formation and subsequent mRNA degradation.

The posttranscriptional processes regulated by these proteins may independently, or in concert, regulate the subcellular distribution of mRNAs, such as *Arc,* in striatal efferent neuron subpopulations. Yet how NMD factors are regulated and expressed within the adult mammalian brain in general, and striatal efferent neurons in particular, is relatively

unexplored, but is of critical interest to advancing our understanding of neuronal signaling *in vivo*. Interestingly, we have recently demonstrated that there is activity-dependent expression of *eIF4A3* mRNA and protein following exposure to a novel environment and that only expression within dorsomedial striatum correlates with behavioral performance on a striatally-mediated T-maze task [268], in a manner very similar to *Arc* mRNA expression [144,269] (Chapter 2). Following behavioral performance on this T-maze task, eIF4A3 protein also colocalizes with *Arc* mRNA within proximal dendrites of striatal neurons [268] (Chapter 2). Furthermore, in an *in vivo* rodent model of cognitive aging, eIF4A3 protein expression within the hippocampus also closely mirrored *Arc* mRNA levels [270]. Given that eIF4A3 critically regulates *Arc* mRNA availability, these findings critically implicate eIF4A3 in contributing to *Arc* mRNA signaling in activated neurons *in vivo*.

Summary of Neuronal RNA Regulation

While it is clear that neurons employ distinct mechanisms to tightly regulate gene expression at the levels of transcription, posttranscriptional processing, nuclear export, cytoplasmic mRNA localization, and local protein synthesis, the contribution of these specific mechanisms are relatively unexplored within the context of striatal neuroplasticity *in vivo*. We have repeatedly demonstrated that striatal efferent neuron subpopulations differentially regulate the cytoplasmic expression of *Arc* mRNA in response to various forms of neuronal activation in both normal and disease states [144,271,145,147]. Yet whether this differential regulation is a feature unique to *Arc* mRNA or reflects an additional difference between striatal efferent neuron subpopulations at the level of general posttranscriptional regulation of mRNA expression is of critical importance to discern so as to improve our understanding of neuroplasticity processes within this essential brain region. As detailed in

the above sections, given that striatal efferent neurons already show divergent expression of neuroanatomical output, dopamine and adenosine receptor subtype expression, neuropeptide expression, and induction of LTP and LTD in response to synaptic stimulation, such observations regarding the differential subcellular localization of this key mRNA essential to the normal regulation of synaptic plasticity processes is less than surprising. However, future investigations into the *in vivo* mRNA regulatory mechanisms contributing to such discrepancies in striatal mRNA processing and subcellular distribution will significantly improve our understanding of the mechanisms contributing to basal ganglia-mediated learning and memory processes. These essential additional investigations will therefore provide direction for future therapeutic development strategies contributing to basal ganglia-mediated pathologies.

Examining Striatal Neuron mRNA Regulation *In Vivo*

Nowhere else in the brain but striatum do different subclasses of projection neurons exist in such a heterogeneous, intermixed fashion while still retaining specific neuroanatomical projection pathways. Thus, discrete examination of the cellular processes contributing to striatal medium spiny neuron function is somewhat complicated by the need to phenotypically identify these neurons prior to examining the subcellular regulatory pathways of interest. Novel cellular analysis tools [141], cell sorting techniques [272], and genetically-modified animal models [273,91] now exist to afford a more complete understanding of the precise signaling differences within striatal efferent neurons. Using phenotypic neuropeptide markers of striatal projection neurons (i.e., *ppe*) and the powerful technique of cellular analysis of temporal localization by fluorescence *in situ* hybridization (catFISH) has allowed our laboratory to begin to specifically explore the regulation and

function of gene expression in greater detail within wild-type, phenotypically identified striatal efferent neurons *in vivo* [144,147].

Herein, we examined how striatal efferent neurons may differentially regulate cytoplasmic *Arc* mRNA availability and localization in the adult, rodent brain *in vivo*. Using the rapid neuronal activation paradigm of spatial exploration of a novel environment [141,144], we examined these mRNA regulatory processes *in vivo*. Then, through the targeted selection of specific candidate mRNAs and RNA binding proteins, we have begun to tease apart a potential mechanism likely contributing to the observed cytoplasmic expression differences of *Arc* mRNA *in vivo*. We first sought to characterize the expression of the core EJC factor, eIF4A3, in the adult mammalian brain *in vivo* and determine whether this EJC factor is activity-regulated within striatum, thus potentially contributing to the activity-dependent expression of *Arc* mRNA following striatally-mediated learning (Chapter 2). Then, using the mRNAs *zif268*, *CaMKIIa,* and *Map2*, which share specifically matched transcriptional activation and cytoplasmic localization characteristics with *Arc* mRNA, we examined whether phenotypic differences in nuclear export, cytoplasmic mRNA trafficking, or mRNA stability could contribute to the cytoplasmic expression differences reported within striatal efferent neurons [147,144,145] (Chapter 3). Finally, we examined how activity-dependent *Arc* mRNA expression is disrupted by pathological conditions, such as following partial monoamine depletion induced by the psychostimulant methamphetamine (METH; Chapter 4), and how such resulting neurodegeneration may participate in neuronal mRNA processing in general (Chapters 3 and 4). Altogether, these specific studies cumulatively demonstrate that striatal efferent neurons engage in different posttranscriptional mRNA processing. Our present findings hold critical implications for basal ganglia-mediated learning and memory processes, as well as pathological conditions of the basal ganglia

[131,274,275,147]. Future investigations into the subcellular processes contributing to such striatal posttranscriptional mRNA regulation differences are critical to more comprehensively understand normal basal ganglia-mediated processing, as well as identify novel therapeutic targets for basal ganglia-mediated pathologies.

Figure 1.1. *Normal basal ganglia circuitry*. The basal ganglia are a collection of subcortical nuclei that mediate normal motor behavior and action selection. The striatum is the key input nucleus of the basal ganglia and receives glutamatergic input from the cortex. Over 95% of neurons within the striatum are divided into two populations of equally expressed, GABAergic spiny efferent neurons: the striatopallidal, "indirect pathway" neurons and the striatonigral, "direct pathway" neurons. These neurons differ in their phenotypic expression of neuropeptides at the cell body level. Striatonigral neurons contain preprotachykinin (*ppt*) / substance P and preprodynorphin / dynorphin. Striatopallidal neurons express preproenkephalin (*ppe*) / met-enkephalin. Furthermore, the striatum receives dopaminergic input from the substantia nigra pars compacta, which leads to very different neuronal activation profiles depending on the striatal efferent neuron subpopulation. Striatonigral neurons express stimulatory $G\alpha_{s/olf}$ protein-linked D1-type dopamine receptors, which are positively coupled to adenylyl cyclase (AC). Conversely, striatopallidal neurons selectively express inhibitory $G\alpha_{i/o}$ protein-coupled D2-type dopamine receptors, which inhibit AC activity. Additionally, striatopallidal neurons selectively express the $G\alpha_{s}$-coupled adenosine 2a receptor (A2A). The striatonigral neurons primarily project to the internal segment of the globus pallidus and substantia nigra pars reticulata, whereas striatopallidal neurons primarily send their axonal projections to the external segment of the globus pallidus. The "direct" pathway neurons selectively provide direct input to the internal segment of the globus pallidus and substantia nigra, and thus reinforce planned movements. Conversely, the "indirect" pathway neurons selectively project to the external segment of the globus pallidus, which in turn projects to the subthalamic nucleus to modulate cortical activity and terminate movements previously directed by activation of the direct pathway.

Normal Basal Ganglia Circuitry

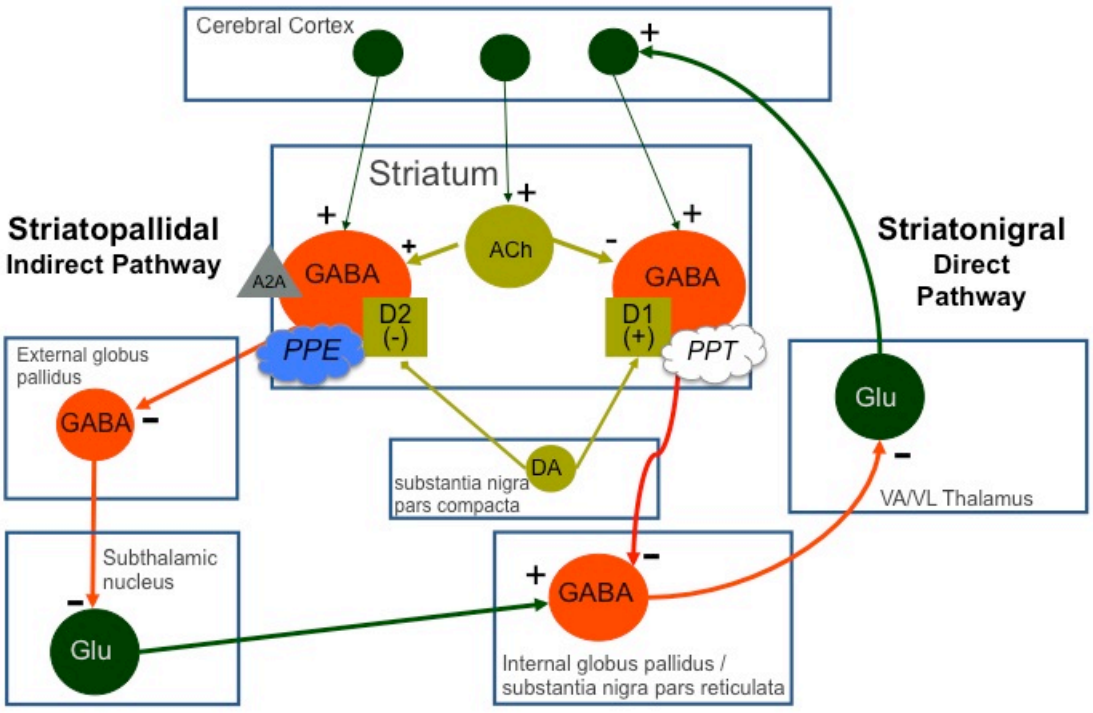


Figure 1.2. *Nuclear mRNA transcription and export.* Following transcriptional activation of *Arc* mRNA by NMDA-type glutamate receptors, ERK-phosphorylation, brain-derived neurotrophic factor (BDNF), or muscarinic acetylcholine receptors (mACh-R), *Arc* pre-mRNA is subject to tight posttranscriptional processing within the nucleus. *Arc* mRNA contains 2 introns within the 3' UTR, and mRNA splicing within the nucleus removes these introns. At the exon-exon junctions, the core components of the exon-junction complex bind to the spliced mRNA. First, the DEAD-Box RNA helicase, eIF4A3, binds to create a scaffold for Magoh-Y14 to bind. These proteins comprise the minimally stable core of the EJC and several other proteins will transiently associate with the EJC during the nuclear lifetime of the mRNP (mRNA-protein complex) as it travels through the nucleus and into the cell cytoplasm. After the core EJC is deposited on target mRNAs, UPF3 associates with the EJC. Additionally, the adaptor protein ALY/REF associates with the EJC. ALY/REF and the TREX component Thoc5 (not illustrated) drive NXF1 into an open conformation able to bind RNA, thus allowing TAP/NXF1 to bind to spliced mRNPs. Recruitment of TAP/NXF1 to spliced mRNPs forms an export-competent mRNA that is now ready to travel via Brownian motion to the nuclear pore complex (NPC) for export out of the nucleus. Prior to nuclear export, the DEAD-Box RNA helicase, UAP56, another member of the TREX complex, remodels the mRNP (some reports suggest that UAP56 dissociates ALY/REF from the mRNP, while others suggest ALY/REF remains associated with the EJC throughout the mRNP lifecycle), although the precise mechanism for nuclear mRNP remodeling by UAP56 is currently unknown. Once an mRNP is presented to the NPC, the mRNP rapidly shuttles through the pore where it meets the DEAD-Box protein DDX19 residing within the cytoplasmic face of the NPC. The ATPase activity of DDX19 is stimulated and rate-limited by GLE1 and its cofactor inositol-hexakisphosphate (IP6), driving conformational changes in DDX19 and thus RNA binding capabilities. DDX19 removes the TAP/NXF1 complex from mRNPs at the cytoplasmic face of the NPC, remodeling the mRNP and permitting the cytoplasmic retention of the mRNP. Importantly, without IP6, DDX19 activity is significantly impaired, causing nuclear mRNA export to be slowed and mRNPs to accumulate in the nucleus.

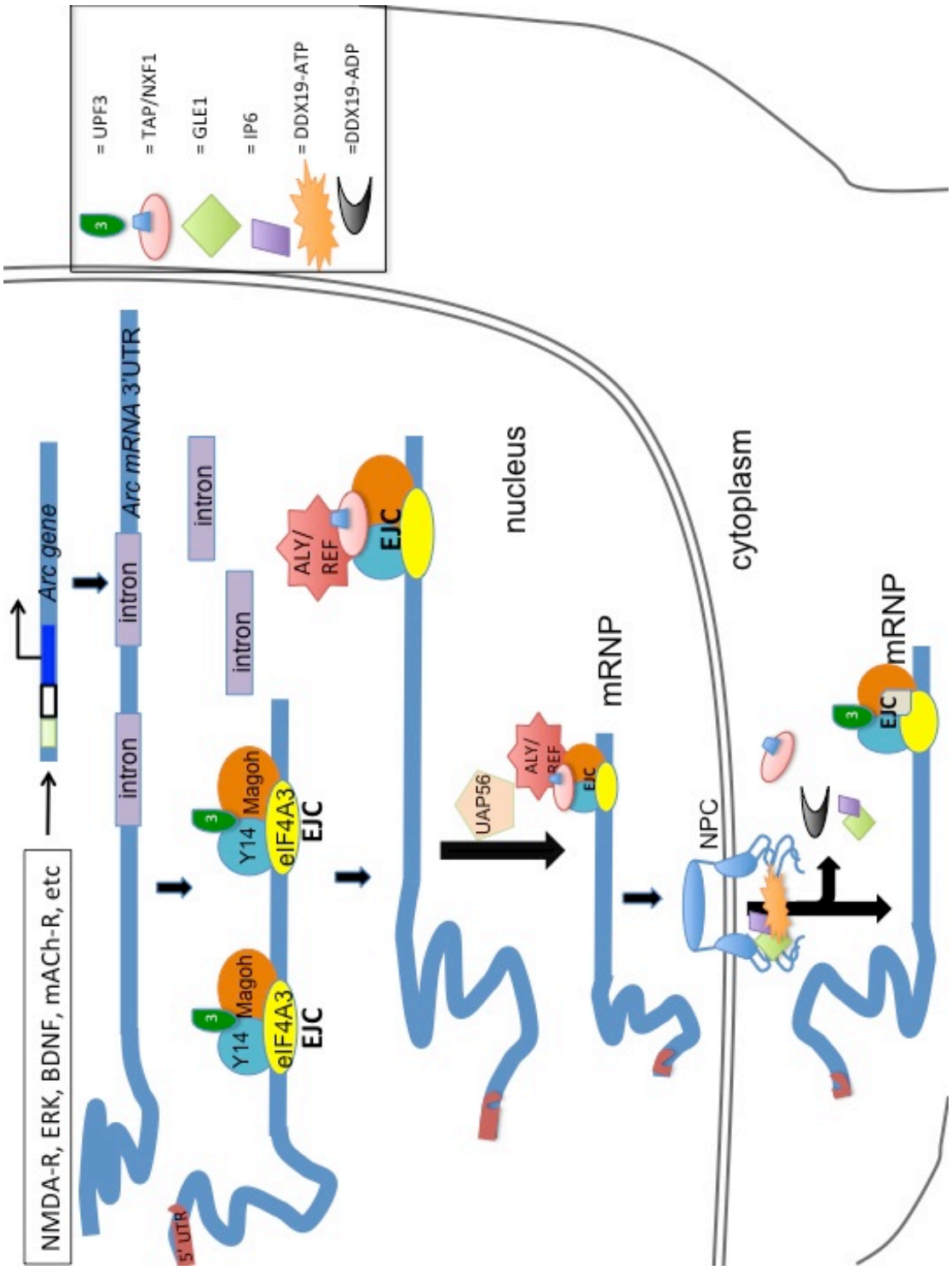
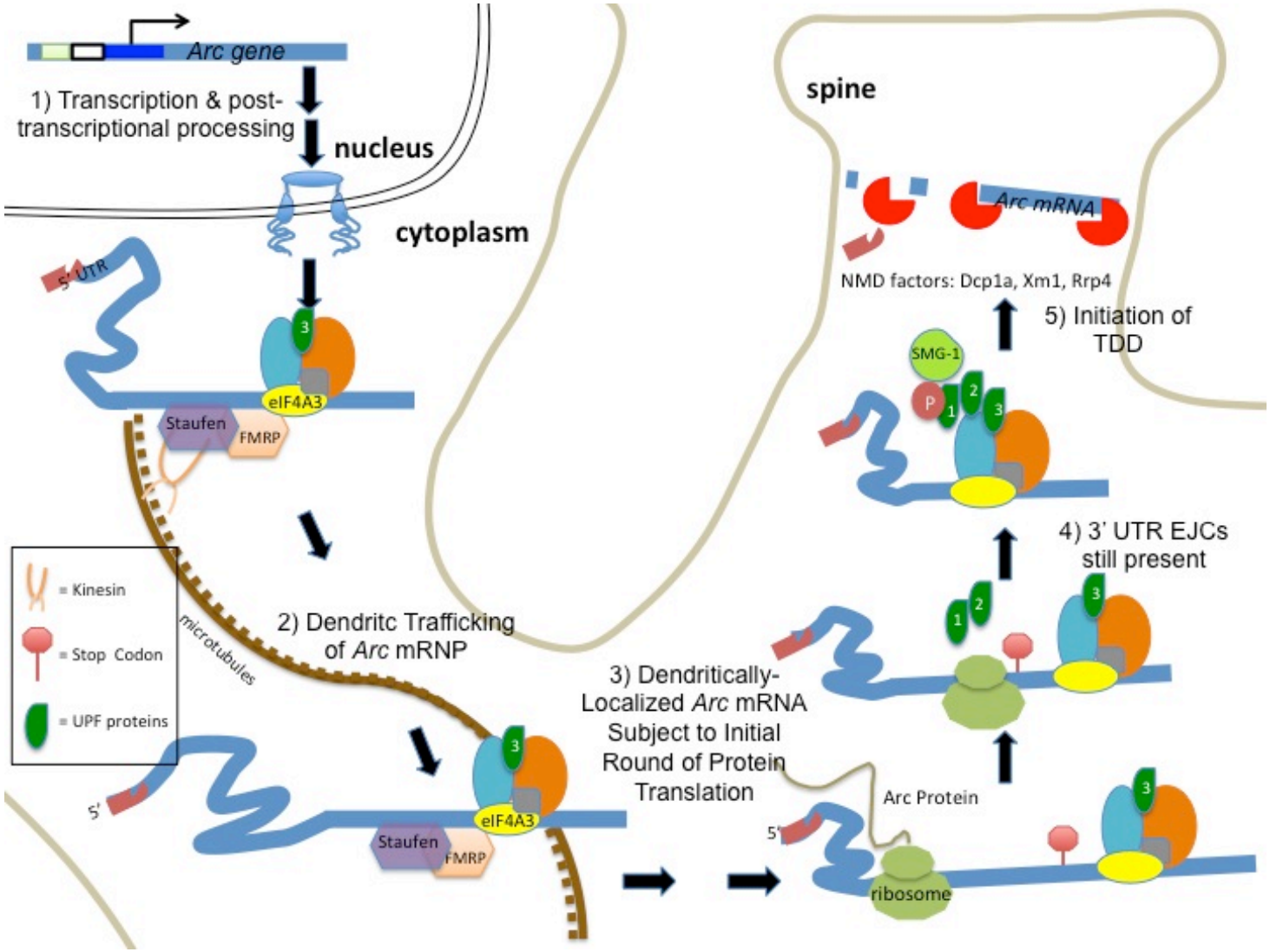

NMDA-R, ERK, BDNF, mACh-R, etc
Arc gene
Arc mRNA 3'UTR
intron
intron
intron
intron
5' UTR
Y14
Magoh
eIF4A3
EJC
ALY/
REF
EJC
nucleus
UAP56
ALY/
REF
EJC
mRNP
cytoplasm
NPC
EJC
mRNP
= UPF3
= TAP/NXF1
= GLE1
= IP6
= DDX19-ATP
=DDX19-ADP

Figure 1.3. *Cytoplasmic mRNA trafficking, localized protein synthesis, and mRNA stability.* 1) Nuclear transcriptional activation, mRNA splicing, and posttranscriptional processing, and nuclear export occurs as described in Figure 1.2. 2) After exiting the nucleus, cargo mRNA-protein granules, such as *Arc*, are trafficked to activated synapses along microtubules by kinesin interacting with mRNA-bound proteins, including Staufen and FMRP. 3) Once mRNAs are transported to dendritic sites, they can be locally translated to proteins essential to LTP and LTD. Dendrites contain all the ribosomal and translational machinery essential to protein synthesis. Normally, as the ribosome proceeds through target mRNAs, it removes any encountered EJCs prior to the stop codon. Then, after the initial round of translation, the translation initiation complex is remodeled to the steady-state translation complex containing the eIF4E protein, thus allowing for continued protein synthesis (not illustrated). 4) However, in the case of *Arc* mRNA, following the first round of protein translation, EJCs remain within the 3'UTR, thus targeting the mRNA to the process of translation-dependent mRNA decay (TDD) mediated by the nonsense mediated mRNA decay (NMD) pathway. 5) These remaining EJC complexes then recruit UPF2 binding to UPF3, and UPF1 binding to UPF2. Upon UPF1 binding, phosphorylation by the SMG-1 kinase initiates mRNA decay to limit cytoplasmic mRNA availability. NMD decay factors thus target the mRNA for degradation: Dcp1 initiates the cleavage of the 5' end cap, which allows for the recruitment of the 5'-to-3' exonuclease Xrn1 and the 3'-to-5' exonuclease Rrp4 that rapidly degrade the mRNA.


Arc gene
1) Transcription & post-transcriptional processing
nucleus
cytoplasm
spine
Arc mRNA
NMD factors: Dcp1a, Xm1, Rrp4
5) Initiation of TDD
SMG-1
P
1
2
3
5' UTR
eIF4A3
Staufen
FMRP
4) 3' UTR EJCs still present
= Kinesin
= Stop Codon
= UPF proteins
microtubules
2) Dendritc Trafficking of Arc mRNP
3) Dendritically-Localized Arc mRNA Subject to Initial Round of Protein Translation
Arc Protein
5'
ribosome

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CHAPTER 2

EXPRESSION OF THE CORE EXON JUNCTION COMPLEX FACTOR EUKARYOTIC INITIATION FACTOR 4A3 IS INCREASED DURING SPATIAL EXPLORATION AND STRIATALLY-MEDIATED LEARNING

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EXPRESSION OF THE CORE EXON-JUNCTION COMPLEX FACTOR EUKARYOTIC INITIATION FACTOR 4A3 IS INCREASED DURING SPATIAL EXPLORATION AND STRIATALLY-MEDIATED LEARNING

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Abstract—Regulation of dendritically localized mRNAs offers an important means by which neurons can sculpt precise signals at synapses. *Arc* is one such dendritically localized mRNA, and it has been shown to contain two exon-junction complexes (EJCs) within its 3′UTR. The EJC has been postulated to regulate cytoplasmic *Arc* mRNA availability through translation-dependent decay and thus contribute to synaptic plasticity. Core proteins of the EJC include eIF4A3, an RNA helicase, and Magoh, which stabilizes the interaction of eIF4A3 with target mRNAs. *Arc* mRNA expression is activity-regulated in numerous brain regions, including the dorsal striatum and hippocampus. Therefore in this study, the *in vivo* expression of these core EJC components was investigated in adult Sprague–Dawley rats to determine whether there are also behaviorally regulated changes in their expression. In the present work, there was no change in the expression of *Magoh* mRNA following spatial exploration, a paradigm previously reported to robustly and reliably upregulate *Arc* mRNA expression. Interestingly, however, there were increases in *eIF4A3* mRNA levels in the dorsal striatum and hippocampus following spatial exploration, similar to previous reports for *Arc* mRNA. Furthermore, there were activity-dependent changes in eIF4A3 protein distribution and expression within the striatum following spatial exploration. Importantly, eIF4A3 protein colocalized with *Arc* mRNA *in vivo*. Like *Arc* mRNA expression, *eIF4A3* mRNA expression in the dorsomedial striatum, but not dorsolateral striatum or hippocampus, significantly correlated with behavioral performance on a striatally-mediated, response-reversal learning task. This study provides direct evidence that a core EJC component, *eIF4A3*, shows activity-dependent changes in both mRNA and protein expression in the adult mammalian brain. These findings thus further implicate eIF4A3 as a key mediator of *Arc* mRNA availability underlying learning and memory processes *in vivo*.

Key words: exon-junction complex, eIF4A3, striatum, T-maze, rat, *Arc/Arg3.1*.

INTRODUCTION

Many dendritically localized mRNAs, including the immediate-early gene *Arc/Arg3.1* (activity-regulated, cytoskeleton-associated protein), are subjected to numerous types of intraneuronal processing, including post-transcriptional regulation and localized protein synthesis (Ule and Darnell, 2006; Panja et al., 2009). Such post-transcriptional processing plays a key role in mediating normal, synapse-specific plasticity (Klann and Dever, 2004; Sutton and Schuman, 2006). The exon-junction complex (EJC) is critical to global mRNA function (Tange et al., 2004), with a role mediating neuronal *Arc* mRNA expression recently coming to light (Giorgi et al., 2007). One postulated mechanism regulating *Arc* mRNA availability is translation-dependent decay (TDD) through the RNA surveillance process of nonsense-mediated mRNA decay (NMD) (Giorgi et al., 2007; Soule et al., 2012), wherein mRNAs can be degraded following the first round of translation if EJCs remain bound to the transcript (Maquat, 2004). *Arc* mRNA is a natural target for TDD due to the two EJCs within the 3′UTR, theoretically leading to tight control of *Arc* mRNA availability and protein synthesis (Giorgi et al., 2007). Thus, the EJC and associated mRNA decay processes could potentially contribute to *Arc* mRNA availability and thus synapse-specific signaling and plasticity.

The minimally stable core of the EJC consists of eukaryotic initiation factor 4A3 (eIF4A3), Magoh/Y14 and MLN51 (Ballut et al., 2005; Tange et al., 2005); absence of eIF4A3 inhibits EJC deposition (Shibuya et al., 2004). As eIF4A3 is the keystone mRNA-binding protein of the EJC, it may act as a brake on cytoplasmic

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Abbreviations: Arc/Arg3.1, activity-regulated, cytoskeleton-associated protein; BDNF, brain-derived neurotrophic factor; CC, caged control; DGlb, lower blade of the dentate gyrus; DGub, upper blade of the dentate gyrus; DIG-UTP, digoxigenin-conjugated UTP; DL, dorsolateral; DM, dorsomedial; eIF4A3, eukaryotic initiation factor 4A3; EJC, exon-junction complex; ELAV, embryonic lethal, abnormal vision protein; FISH, fluorescence *in situ* hybridization; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; NMD, nonsense-mediated mRNA decay; PBS-T, PBS/0.1% Triton-X; PBS-TB, PBS/0.1% Triton-X/0.1% Bovine Serum Albumin; TDD, translation-dependent decay; TNT, TBS/0.05% Tween-20; TSA, tyramide signal amplification.

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Arc mRNA availability (Giorgi et al., 2007) and thereby potentially play a key role in neuroplasticity. Dynamic regulation of eIF4A3 may thus offer an intriguing means to modulate synaptic plasticity processes in the adult mammalian brain due to its core role in EJC formation (Shibuya et al., 2004; Tange et al., 2005) and known association with mRNAs critical to neuronal function (Giorgi et al., 2007). However, whether eIF4A3 mRNA and protein expression changes in response to neuronal activation in the adult mammalian brain *in vivo* has yet to be fully explored and was thus the goal of the present work.

In the present study, we demonstrate activity-dependent increases in the expression of *eIF4A3* mRNA, but not the expression of another EJC component *Magoh*, in the adult rodent CNS. We also report brain-region specific relation of *eIF4A3* mRNA expression to behavioral indices of new learning, consistent with previous observations for *Arc* mRNA (Guzowski et al., 1999; Daberkow et al., 2007, 2008). The present findings thus suggest that the expression of *eIF4A3* is activity-regulated and that *eIF4A3* expression in brain regions being engaged in a particular learning task correlates with that learning. These results thus provide evidence that not only do neurons regulate many effector genes critical for plasticity processes, they also dynamically regulate the regulatory elements contributing to such signaling in the adult mammalian brain *in vivo*.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC; 275–300 g) were singly housed in tub cages in a room controlled for temperature and lighting (12:12 h). Animal care and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 8th ed.) and were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Novel spatial exploration

Rats were divided into five experimental groups of 4–10 rats each. Rats were handled prior to the experiment to familiarize them to the experimenter and handling. Rats were exposed to a 2-ft × 2-ft plastic tub, novel spatial environment, a paradigm known to robustly and reliably induce *Arc* mRNA and other immediate-early gene expression, as previously described (Guzowski et al., 1999; Chawla et al., 2005; Vazdarjanova et al., 2006; Daberkow et al., 2007). Rats were removed from their home cage after having been isolated in the cage for 24 h. Caged control (CC) rats (n = 10) were sacrificed immediately upon removal from the home cage. The ''5 min'' group rats (n = 10) were exposed to the novel environment for 5 min and then immediately sacrificed; ''30 min'' group rats (n = 10) were exposed for 5 min to the novel environment and then returned to the home cage for 25 min before sacrifice; ''60 min'' group rats (n = 4) were exposed for 5 min to the novel environment and then returned to the home cage for 55 min before sacrifice. Animals were sacrificed by exposure to CO_2, decapitated and brains immediately removed and flash-frozen in 2-methylbutane (Mallinckrodt Baker, Phillipsburg, NJ) chilled on dry ice.

T-maze, response-reversal learning task

A separate group of rats (n = 11) was habituated to the T-maze and experimenter as previously described (Daberkow et al., 2007, 2008; Pastuzyn et al., 2012). After T-maze habituation, the turn bias of each rat was determined, and acquisition training on the T-maze proceeded as previously described (Daberkow et al., 2007; Pastuzyn et al., 2012). During the response-reversal task, rats were rewarded for turning in the opposite direction from acquisition with task completion being when the rat reached criterion (9/10 correct consecutive turns). Each trial on this task took approximately 1 min. Five minutes after reaching criterion, animals were sacrificed and brains collected as described above. As with the novel spatial exploration task, CC rats (n = 11) associated with this experiment were immediately sacrificed upon removal from their home cage.

Tissue preparation

Striatal and hippocampal sections (Bregma: +1.2–1.5 mm and −2.8–3.3 mm, respectively (Paxinos and Watson, 1998)) were cryosectioned at 12-μm (Cambridge Instruments, Bayreuth, Germany) and thaw-mounted onto SuperFrost Plus slides (VWR, Batavia, IL). Slides to be directly compared were processed in parallel. Slides were postfixed as previously described (Ganguly and Keefe, 2001), air-dried and stored at −20 °C until histochemical processing.

Fluorescent immunohistochemistry

Striatal sections to be labeled for eIF4A3 protein were washed 2 × 5 min in PBS/0.1% Triton-X (PBS-T), blocked for 1 h with PBS/0.1% Triton X/0.1% Bovine Serum Albumin (PBS-TB), and then washed 2 × 5 min in PBS-T. Protein was then detected with rabbit anti-eIF4A3 antibody (Abcam, Cambridge, MA) at a concentration of 5 μg/mL overnight at 4 °C. Slides were then washed 2 × 5 min in PBS-T followed by incubation with donkey anti-rabbit Alexa Fluor-488 (1:1000; Invitrogen, Carlsbad, CA) in PBS-TB for 2 h at 24 °C. Slides were washed 2 × 5 min in PBS-T and coverslipped with Prolong Gold mounting media with DAPI nuclear counterstain (Invitrogen, Carlsbad, CA).

Radioactive *in situ* hybridization

Full-length rat *eIF4A3* and mouse *Magoh* (93% sequence homology to rat) cDNA-containing vectors were purchased for plasmid isolation (eIF4A3 [GenBank: BC105875.1] clone ID 7120292; Magoh [GenBank: BC018176.1] clone ID 3587774; Open Biosystems, Huntsville, AL). The cDNAs were linearized (*EcoRI*; Roche Applied Science, Indianapolis, IN), and probes transcribed with [^{35}S]-UTP or [^{33}P]-UTP (Perkin-Elmer, Waltham, MA) with T7 RNA polymerase (Roche Applied Science, Indianapolis, IN). *In situ* hybridization was performed as previously described (Ganguly and Keefe, 2001).

Dual Arc fluorescent *in situ* hybridization and eIF4A3 immunohistochemistry

Co-expression of *Arc* mRNA and eIF4A3 protein in the dorsal striatum was determined by combined fluorescence *in situ* hybridization histochemistry (FISH) for *Arc* mRNA and eIF4A3 protein, as previously described for *Arc* FISH (Daberkow et al., 2007, 2008) and as described above (Section Fluorescent immunohistochemistry) for eIF4A3 protein with minor modifications to the buffers (TBS/0.05% Tween-20 (TNT) buffer: 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20). A full-length ribonucleotide probe complementary to *Arc* mRNA (Lyford et al., 1995) was synthesized from cDNAs using digoxigenin-UTP (DIG-UTP) with T7 RNA polymerase and

DIG-UTP RNA labeling kit (Roche) (Daberkow et al., 2007, 2008). Slides were hybridized with *Arc* ribonucleotide probe overnight (12–18 h) in a humid chamber at 56 °C. Once removed, slides were vigorously washed at 24 °C four times in 2× SSC buffer (0.15 M NaCl with 0.015 M sodium citrate). Slides were then washed in ribonuclease A (RNase A; 10 μg/mL; Roche Applied Science) in 2× SSC for 15 min. After incubation with RNase A, slides were washed 5 min in 2× SSC, then 4 × 20 min in 0.2× SSC at 24 °C. Endogenous peroxidase activity was then quenched with 2% H_2O_2 for 15 min, slides were washed 2 × 5 min in TNT buffer, and then slides were incubated for 2 h at RT with an anti-digoxigenin antibody (1:1000) coupled to horseradish peroxidase (HRP; Roche). The *Arc* probe was detected by cyanine-3 (cy-3) tyramide signal amplification (tyramide signal amplification (TSA) Plus; Perkin-Elmer). After detection of the DIG-labeled *Arc* ribonucleotide probe, slides were washed in 2 × 5 min in TNT and eIF4A3 protein detected as above (Section Fluorescent immunohistochemistry). The next day, slides were washed 2 × 5 min in TNT, incubated for 2 h RT with a goat anti-rabbit-HRP antibody (Millipore, Billerica, MA), then washed 2 × 5 min in TNT. The eIF4A3 protein signal was detected by fluorescein TSA (Perkin-Elmer) then washed 2 × 5 min in TNT. Finally, slides were coverslipped with Prolong Gold mounting media with DAPI (Invitrogen).

Image acquisition and analysis

To determine eIF4A3 protein expression in the dorsomedial (DM) striatum (+1.2–1.5 mm bregma), 0.6 mm^2 fields from two striatal sections per animal were imaged on a Leica DM4000B fluorescent microscope at 40×. Each image was analyzed using *ImageJ* (NIH) with the inverted LUT threshold set to 15 to remove background. The mean gray value and percent of the total field area with eIF4A3 signal were measured.

Film autoradiograms of sections processed for radioactive *in situ* hybridization were digitized (*ImageJ*). For both *eIF4A3* and *Magoh* mRNAs, four sections per animal were imaged from the dorsal striatum (rostral (+1.2–1.5 mm bregma) and middle (+0.5 mm bregma)) and dorsal hippocampus (−2.8–3.3 mm bregma). Film autoradiograms were analyzed using *ImageJ* (Ganguly and Keefe, 2001). The mean gray value of white matter was subtracted from the mean gray value of the regions of interest. Mean gray values for each animal were then normalized to the average signal in the CC group.

Combined FISH/immunohistochemistry images (105.5 μm × 105.5 μm) in the DM striatum (+1.2–1.5 mm bregma; Fig. 4A–D) were captured under 2× zoom magnification with an FV1000 confocal laser-scanning microscope (Olympus) with motorized stage (Prior Scientific) using a 60×, 1.45 NA oil-immersion lens (plan APO) and 405-nm Diode, 488-nm Ar, and 543-nm HeNe lasers (Daberkow et al., 2007, 2008). Areas of analysis were *z*-sectioned in 0.5-μm-thick optical sections.

Statistical analyses

Expression of mRNAs and protein in rats after spatial exploration were compared using one-way ANOVAs followed by *post hoc* Dunnett's tests. Levels of *eIF4A3* mRNA expression following response-reversal learning were compared to CC levels using two-tailed *t*-tests and also correlated with performance.

RESULTS

Spatial exploration and expression of EJC components eIF4A3 and Magoh

Initially, we characterized the *in vivo* expression of the EJC factor *eIF4A3* in the dorsal striatum of rats following spatial exploration of a novel environment (Fig. 1). *eIF4A3* expression in the dorsal striatum increased above that in CC rats following novel environment exploration (Fig. 1A; "5 min" = 114.7% ± 8.0 of CC; "30 min" = 123.0% ± 3.9 of CC; "60 min" = 102.3% ± 14.4 of CC; $F_{(3,35)}$ = 3.29, p = 0.03). *Post-hoc* analysis revealed a significant increase over CC animals in the "30 min" group (p = 0.03; Fig. 1D,E). We then asked whether spatial exploration was associated with an increase in *Magoh* expression, which would suggest global changes in the expression of EJC factors. Although *Magoh* was also basally expressed, we did not detect any significant increases in *Magoh* mRNA expression in animals that engaged in the spatial exploration task ("5 min" = 98.7% ± 1.64 of CC; "30 min" = 95.9% ± 1.93 of CC; "60 min" = 98.3% ± 4.91 of CC; $F_{(3,20)}$ = 0.73, p > 0.05; Fig. 1B).

Given that *Arc* mRNA expression is increased in an activity-dependent manner in numerous brain regions (Guzowski et al., 1999; Chawla et al., 2005; Daberkow et al., 2007), we examined whether activity-dependent *eIF4A3* mRNA expression was similarly broadly distributed in the brains of rats subsequent to spatial exploration of a novel environment. As in the dorsal striatum, there was a time-dependent increase in *eIF4A3* mRNA expression in the dorsal hippocampus (Fig. 2A–D), including the CA1 ("5 min" = 108.2% ± 2.4, "30 min" = 111.4% ± 3.0, "60 min" = 128.5% ± 8.2; $F_{(3,32)}$ = 3.18, p = 0.04; Fig. 2A) and CA3 ("5 min" = 106.5% ± 1.5, "30 min" = 108.3% ± 2.1, "60 min" = 119.0% ± 5.8; $F_{(3,32)}$ = 2.97, p = 0.05; Fig. 2B) subregions. *Post-hoc* analysis revealed significant increases in *eIF4A3* mRNA expression over CC animals in the CA1 of the "30 min" group (p = 0.02) and a trend for increases in expression over CC animals in the "60 min" group (p = 0.08). Likewise, *post hoc* analysis of the data from CA3 revealed a significant increase over CC animals in the "30 min" group (p = 0.05). Similar to previous reports for novelty-induced *Arc* mRNA expression in the dentate gyrus (DG) subregion of the hippocampus (Chawla et al., 2005), there was a time-dependent increase in *eIF4A3* mRNA expression in the upper blade of the dentate gyrus (DGub; $F_{(3,32)}$ = 3.22, p = 0.04). *Post-hoc* analysis revealed significant increases in eIF4A3 mRNA expression in the DGub of the "5 min" (116.4% ± 4.6, p = 0.05) and "30 min" (116.5% ± 3.4, p = 0.05; Fig. 2C) groups relative to the CC group, as well as a trend for increase in expression in the "60 min" group (119.7% ± 3.4, p = 0.07). Conversely, there was no change in eIF4A3 expression in the lower blade of the dentate gyrus (DGlb $F_{(3,32)}$ = 1.37, p = 0.3; Fig. 2D) at any time point.

As with the striatum, there were no significant increases in *Magoh* mRNA expression in any regions of the hippocampus following spatial exploration (Fig. 2E–H; CA1 "5 min" = 109.9% ± 5.3, "30 min" = 105.0 ± 3.1, "60 min" = 115.8% ± 4.1; $F_{(3,31)}$ = 1.15, p > 0.05; CA3 "5 min" = 98.3% ± 4.2, "30 min" = 103.0% ± 3.6, "60 min" = 98.4% ± 4.1; $F_{(3,31)}$ = 0.65, p > 0.05; DGub "5 min" = 103.4% ± 4.0, "30 min" = 106.4% ± 1.8, "60 min" = 92.6% ± 3.2; $F_{(3,18)}$ = 0.35, p > 0.05; DGlb "5 min" = 96.8% ± 5.4, "30 min" = 100.3 ± 4.7, "60 min" = 87.1% ± 5.0; $F_{(3.18)}$ =

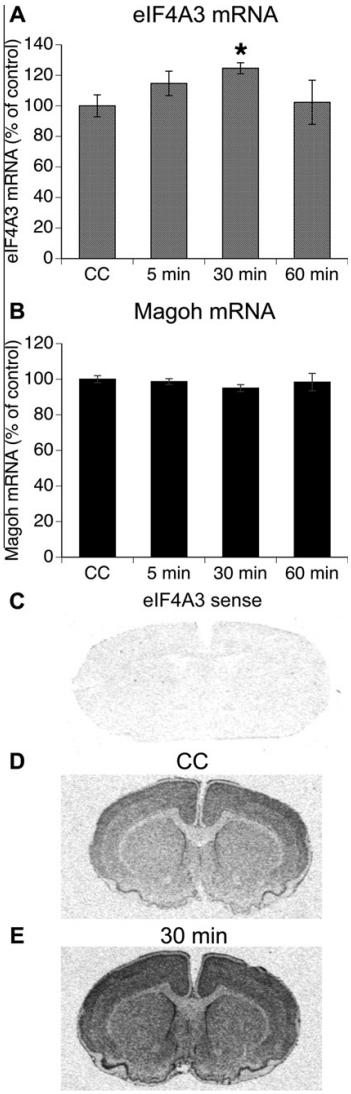

Fig. 1. Expression of EJC components in dorsal striatum of rats engaged in spatial exploration for 5 min. (A) Mean expression (arbitrary gray value) of *eIF4A3* mRNA (±SEM; n = 4–10/group) in dorsal striatum analyzed via radioactive *in situ* hybridization histochemistry and expressed as a percent of basal values in caged control (CC) animals. Rats in the CC group were sacrificed immediately upon removal from their home cage. Rats in the remaining groups explored a novel spatial environment (see Experimental procedures) for 5 min and were then either sacrificed immediately (''5 min'' group) or returned to the home cage for 25 min before sacrifice (''30 min'' group); or 55 min before sacrifice (''60 min'' group). *Significantly different from CC (p = 0.03). (B) Mean expression (arbitrary gray value) of *Magoh* mRNA (±SEM; n = 4–7/group) in dorsal striatum analyzed via radioactive *in situ* hybridization histochemistry and expressed as a percent of basal values in CC animals. (C) A sense ribonucleotide probe for *eIF4A3* gave no signal. (D) Striatal section from a CC rat labeled with the anti-sense ribonucleotide probe for *eIF4A3*. (E) Striatal section from a ''30 min'' group rat labeled with the anti-sense ribonucleotide probe for *eIF4A3*.

1.38, $p > 0.05$). Thus, there were activity-related increases in *eIF4A3*, but not *Magoh*, mRNA in both the dorsal striatum (Fig. 1) and dorsal hippocampus (Fig. 2). The activity-related expression of *eIF4A3* is similar to the pattern of exploration-induced *Arc* mRNA expression previously reported, including time-dependent increases in both the dorsal striatum (Daberkow et al., 2007) and CA1, CA3, and DGub subregions of the hippocampus (Guzowski et al., 1999; Chawla et al., 2005; Vazdarjanova et al., 2006).

Activity-related increase in eIF4A3 protein distribution in striatum

To further assess the activity-dependent regulation of *eIF4A3*, we used fluorescence immunohistochemistry to examine the expression of eIF4A3 protein after animals engaged in spatial exploration of a novel environment (Fig. 3). CC animals exhibited low basal distribution of eIF4A3 protein (percent of total field area with signal) but high signal intensity (mean gray value of pixels). Following exploration, there was a significant time-dependent increase in the percent of the total field area with signal ($F_{(4,37)}$ = 4.95, p = 0.003; Fig. 3A), with the ''30 min'' group showing increased distribution of the eIF4A3 signal relative to the CC group ($p < 0.01$). This distribution returned to basal levels by 60 min (Fig. 3A), such that expression within the ''60 min'' group was not significantly different from the CC group ($p > 0.9$). Furthermore, there was a significant time-dependent effect on eIF4A3 protein signal intensity (mean gray value) within the striatum ($F_{(4,37)}$ = 5.21, p = 0.002; Fig. 3B). *Post-hoc* analysis revealed a significant decrease in the signal intensity in the ''30 min'' group (p = 0.02) relative to the CC group. As was the case for the distribution of the eIF4A3 signal, the increase in signal intensity returned to basal levels by 60 min, such that expression in the ''60 min'' group was not significantly different from the CC group (p = 0.7). Correlating the measurement of percent field area with eIF4A3 signal to the signal intensity (mean gray value of pixels) revealed a strong inverse correlation (R^2 = 0.89, $p < 0.0001$; Fig. 3C), suggesting that there is activity-dependent movement of this protein together with mRNA granules, such as *Arc* (Kanai et al., 2004), during periods of neuronal activation rather than *de novo* synthesis of protein.

Colocalization of eIF4A3 protein with Arc mRNA

To examine whether *Arc* mRNA and eIF4A3 protein interact *in vivo*, we performed double-label detection of *Arc* mRNA and eIF4A3 by combined FISH/fluorescent immunohistochemistry followed by confocal imaging in a subset of rats (n = 3) following response-reversal learning on the T-maze (Fig. 4). Areas of *Arc* mRNA and eIF4A3 protein colocalization are found throughout the DM striatum under these conditions (Fig. 4A). To estimate the extent of colocalization, two separate image fields from each T-maze-trained rat were analyzed. On average, 43.5% ± 0.02 of *Arc*

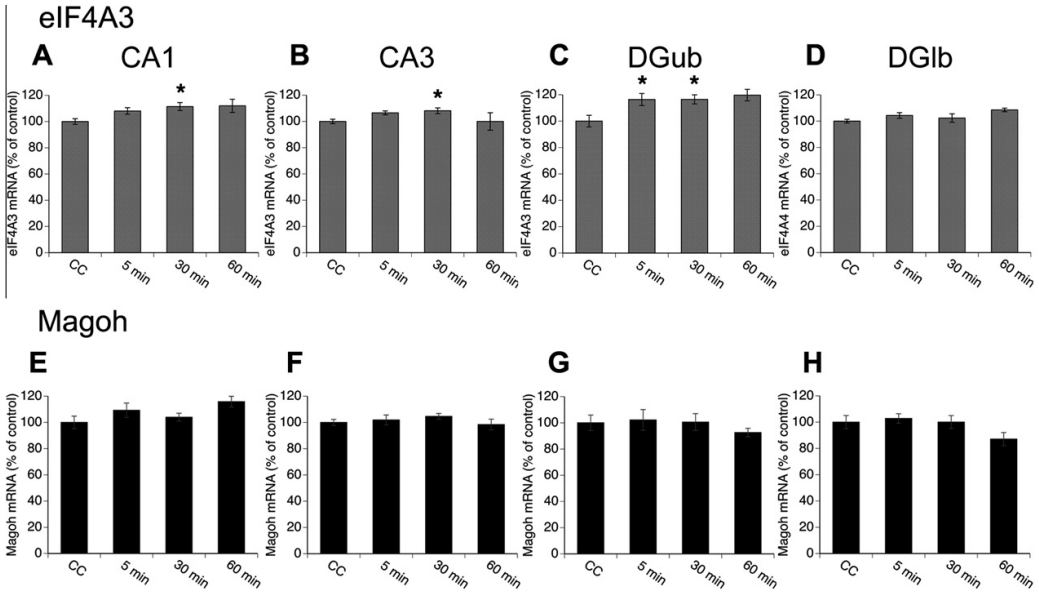

Fig. 2. Expression of EJC components in dorsal hippocampus of rats engaged in spatial exploration for 5 min. (A–D) Mean expression (arbitrary gray value) of *eIF4A3* mRNA (±SEM; $n = 4$–10/group) in the CA1 (A), CA3 (B), upper blade of the dentate gyrus (DGub; C), and lower blade of the dentate gyrus (DGlb; D) subregions of dorsal hippocampus analyzed via radioactive *in situ* hybridization histochemistry and expressed as a percent of basal values in caged control (CC) animals. *Significantly different from CC. (E–H) Mean expression (arbitrary gray value) of *Magoh* mRNA (±SEM; $n = 10$/group) in CA1 (E), CA3 (F), DGub (G), and DGlb (H) analyzed via radioactive *in situ* hybridization histochemistry and expressed as a percent of basal values in CC animals.

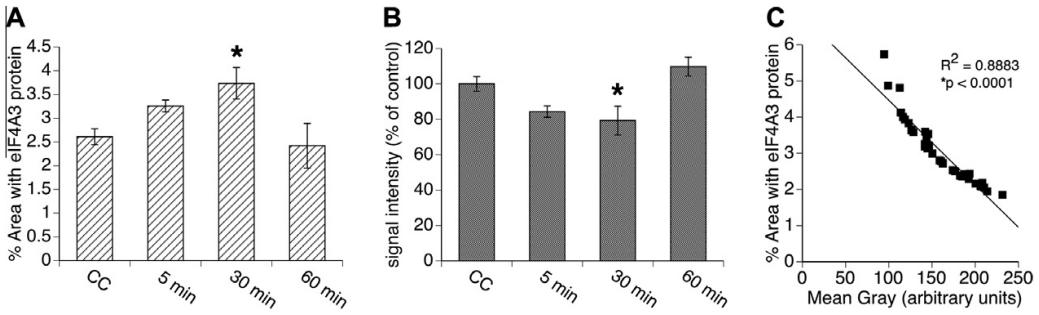

Fig. 3. Expression of eIF4A3 protein in dorsal striatum of rats engaged in spatial exploration for 5 min. (A) Mean (±SEM, $n = 4$–10/group) eIF4A3 protein distribution in dorsal striatum measured as percent of total image area with signal (i.e. percentage of total pixel area in the field with eIF4A3-labeled pixels). *Significantly different from caged controls (CC; $p < 0.01$). (B) Signal intensity (average gray area), expressed as mean percent of control (±SEM, $n = 4$–10/group), of eIF4A3 protein-labeled pixels in dorsal striatum *Significantly different from CC ($p = 0.02$). (C) Significant inverse correlation ($p < 0.05$) between percent of total field with eIF4A3 protein signal above threshold and the average signal intensity (mean gray value) of the labeled pixels.

mRNA-positive puncta colocalized with eIF4A3 protein-positive puncta outside of the somatic compartment, whereas 33.9% ± 0.2 of eIF4A3 protein-positive puncta colocalized with *Arc* mRNA-positive puncta. A previous study reported that ~59% of *Arc* mRNA-positive puncta colocalized with eIF4A3 protein-puncta, whereas 29% of eIF4A3 protein-positive puncta colocalized with *Arc* mRNA-positive puncta *in vitro* following 6-h incubation of cultured hippocampal neurons with brain-derived neurotrophic factor (BDNF) (Giorgi et al., 2007), Additionally, another study reported that the neuron-specific RNA-binding protein Smaug-1 also showed 40–60% colocalization with *CaMKIIa* mRNA following NMDA receptor stimulation in cultured hippocampal neurons *in vitro*, with this protein contributing to *CaMKIIa* mRNA availability and activity-dependent protein synthesis (Baez et al., 2011). Thus, our analysis of the *in vivo* colocalization of *Arc* and eIF4A3 following learning is in line with previous reports for RNA-binding proteins colocalizing with target mRNAs following various pharmacological stimulation paradigms *in vitro*.

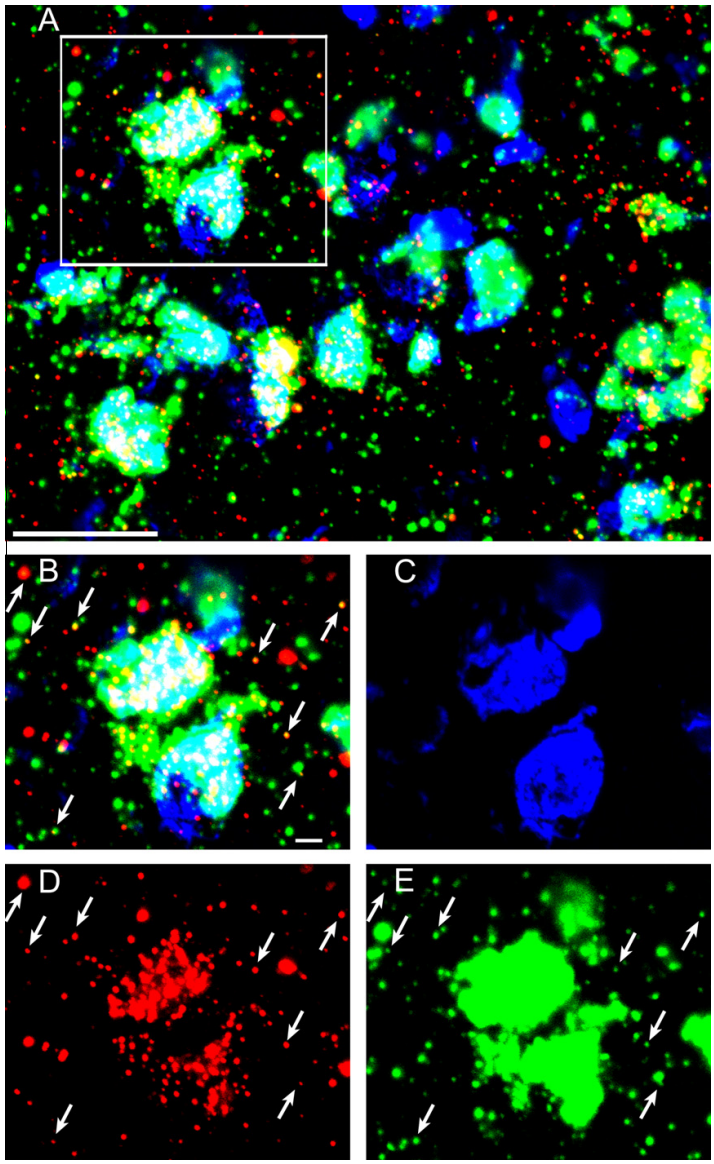

Fig. 4. *Arc* mRNA colocalizes with eIF4A3 protein *in vivo* following response-reversal learning on a T-maze. (A) Representative photomicrograph of *Arc* mRNA *in situ* hybridization histochemical staining (red) and eIF4A3 protein immunofluorescence (green) in the dorsal striatum of a rat sacrificed 5 min after reaching criterion on a striatally mediated, response-reversal learning task (see Experimental procedures). Scale bar = 20 μm. (B–E) Higher magnification images of the region delineated in the box in (A) showing colocalization of eIF4A3 protein expression and *Arc* mRNA colocalization (B) and the individual channels for the DAPI nuclear counter stain (C), *Arc* mRNA (D), and eIF4A3 protein (E). Arrows highlight points of colocalization of *Arc* mRNA and eIF4A3 signal. Scale bar in B–E = 2 μm.

Correlation between eIF4A3 mRNA expression and striatally-mediated learning

Previously, we reported that *Arc* mRNA expression in the DM, but not dorsolateral (DL), striatum of normal rats correlates with trials to criterion on a T-maze-based, response-reversal learning task (Daberkow et al., 2007). Furthermore, knockdown of *Arc* mRNA in the DM striatum impairs consolidation of reversal learning on this task (Pastuzyn et al., 2012). Thus, we examined whether *eIF4A3* mRNA expression correlates with behavioral performance. Similar to our prior observations with *Arc* mRNA (Daberkow et al., 2007,

2008), *eIF4A3* mRNA expression significantly increased in the DM and DL striatum of animals performing on the T-maze relative to CC rats (Fig. 5A; CC–DM: 34.9 ± 1.9; DL: 35.5 ± 2.38; T-maze–DM: 43.3 ± 1.1, $t_{(10)} = 6.63$, $p = 0.0001$; DL: 44.0 ± 1.2, $t_{(10)} = 6.3$, $p = 0.0001$). However, only *eIF4A3* mRNA expression in the DM striatum ($R^2 = 0.39$; $p = 0.04$; Fig. 5B) significantly correlated with behavioral performance (i.e. trials to criterion); *eIF4A3* mRNA expression in the DL striatum ($R^2 = 0.02$; $p > 0.1$; Fig. 5C) or any subregion of the dorsal hippocampus (CA1, $R^2 = 0.22$; $p > 0.1$; CA3, $R^2 = 0.11$; $p > 0.1$; DGub $R^2 = 0.16$; $p > 0.1$; DGlb $R^2 = 0.11$; $p > 0.1$; Fig. 5D, E) did not correlate with behavioral performance.

DISCUSSION

We presently demonstrate increased expression of the EJC factor *eIF4A3* in the adult rodent CNS under conditions of spatial exploration and striatally-mediated response-reversal learning. The level of *eIF4A3*, but interestingly not *Magoh*, mRNA increased in an activation-related manner across multiple brain regions, and expression of *eIF4A3* mRNA in the DM striatum correlated with behavioral performance on a striatally-based response-reversal learning task. Furthermore, eIF4A3 protein colocalized with *Arc* mRNA *in vivo* following striatally-mediated learning. Given the complex signaling required of neurons undergoing activity-dependent synaptic modifications, it is conceivable that such plasticity requires activity-dependent expression of *eIF4A3* to facilitate normal mRNA quality control through NMD (Chang et al., 2007; Shyu et al., 2008) and to regulate the translation and decay of plasticity-related mRNAs, such as *Arc* (Giorgi et al., 2007; Soule et al., 2012). The present observations are the first to demonstrate activity-dependent changes in *eIF4A3* mRNA and protein levels in the adult mammalian brain *in vivo*, further implicating *eIFAA3* as a mediator of activity-dependent neuroplasticity processes.

eIF4A3 likely plays an important role in neuronal mRNA processing given its core role in EJC formation and function (Shibuya et al., 2004; Ballut et al., 2005). Numerous aspects of post-transcriptional mRNA processing are mediated by the EJC, including nucleocytoplasmic shuttling (Shibuya et al., 2004), cytoplasmic translational control (Diem et al., 2007), and NMD/TDD (Maquat, 2004). Specifically, continued presence of the EJC on spliced mRNAs following the first round of protein translation can initiate mRNA decay by NMD (Maquat, 2004; Shibuya et al., 2004), thereby tightly limiting mRNA availability. Given the exclusive role that eIF4A3 plays in EJC formation (Chan et al., 2004; Shibuya et al., 2004) and NMD (Ferraiuolo et al., 2004), it is likely a critical component of mRNA stability underlying normal neuronal signaling in the adult mammalian CNS.

Numerous neuronal mRNAs are dendritically targeted for local protein synthesis, indicating a need for tight post-transcriptional and translational regulation of these mRNAs to coordinate neuronal signaling (Ule and Darnell, 2006; Goldie and Cairns, 2012). The EJC, and eIF4A3 specifically, may thus allow neurons to regulate the expression of specific effector mRNAs, such as *Arc*, through NMD/TDD or through other post-transcriptional processes involved in the trafficking of such effector mRNAs to or translational control of those mRNAs at synapses. Unlike other eIF4A isoforms (Li et al., 1999), which are components of the eIF4F translation initiation complex (Klann and Dever, 2004), eIF4A3 plays a distinct role in mRNA stability. Understanding whether these other eIF4A isoforms also show activity-dependent changes in expression will help clarify the precise contribution made by the EJC and NMD vs. translational regulation in determining synaptic plasticity processes. However, given the known role of eIF4A3 in mRNA regulation and its activity-related expression, mRNA stability processes mediated by NMD may act as a potential means by which neurons can regulate response to synaptic stimuli.

As demonstrated by others (Giorgi et al., 2007; Soule et al., 2012) and now us (Fig. 4), one potential means for *eIF4A3* to contribute to synaptic plasticity is through interaction with and regulation of *Arc* mRNA. As eIF4A3 is critical to the EJC (Chan et al., 2004; Shibuya et al., 2004; Ballut et al., 2005), it may act as a brake on cytoplasmic *Arc* mRNA availability through NMD (Giorgi et al., 2007). *Arc* is a target for NMD due to the two EJCs within the 3′UTR, leading to tight control of Arc protein synthesis (Giorgi et al., 2007; Soule et al., 2012). Knockdown of eIF4A3 increased *Arc* mRNA and protein levels in rat hippocampal somata and dendrites *in vitro* and increased miniature excitatory postsynaptic current (mEPSC) amplitude and synaptic metabotropic glutamate receptor 1 (mGLUR1) levels (Giorgi et al., 2007), indicating that eIF4A3 can directly affect *Arc*-dependent synaptic plasticity. This present work demonstrates time-dependent changes in eIF4A3 protein distribution and signal intensity following exploration of a novel environment. Additionally, eIF4A3 protein colocalized with *Arc* mRNA in the dorsal striatum following striatally-mediated learning (Fig. 4), similar to previous reports *in vitro* (Giorgi et al., 2007). Thus, one potential explanation for the observed increase in distribution of eIF4A3 protein is that neuronal activation associated with spatial exploration or engagement on a learning and memory task induces *de novo* transcription of effector mRNAs, such as *Arc* (Guzowski et al., 1999; Daberkow et al., 2007), which in turn leads to the formation of eIF4A3-containing protein-mRNA granules (Kanai et al., 2004) that then distribute out into dendrites. Such greater distribution of the eIF4A3-containing granules throughout the neuropil may then result in the lower signal intensity in any given set of labeled pixels. However, we cannot presently rule out the possibility that the observed changes in eIF4A3 protein expression also reflect *de novo* eIF4A3 protein synthesis. Many plasticity-associated mRNAs, including *CaMKIIα* (Ouyang et al., 1997), *zif268* (Zangenehpour and Chaudhuri, 2002) and even *Arc* (Vazdarjanova et al., 2006; Niere et al., 2012), demonstrate rapid protein synthesis in response to stimuli. Thus, while it was somewhat surprising that the percent of the total

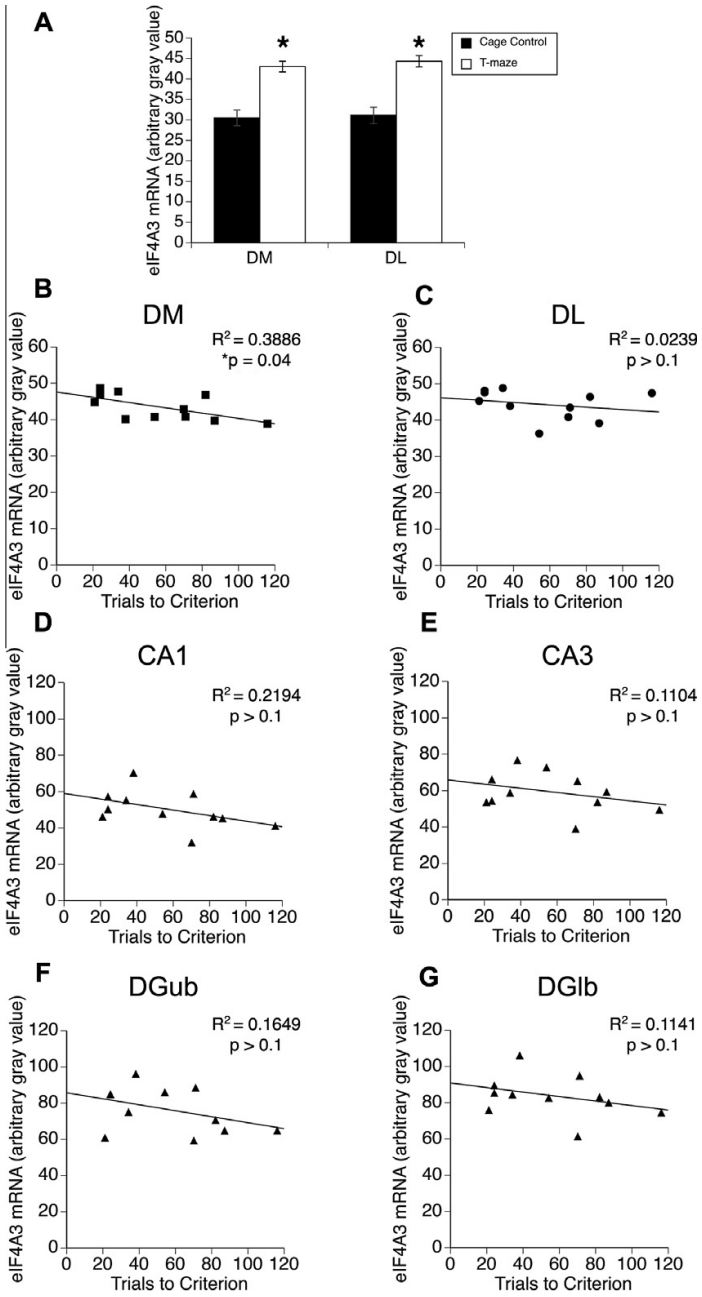

Fig. 5. Expression of *eIF4A3* mRNA in the brains of rats undergoing response-reversal learning. (A) Rats trained to perform on a response-reversal learning task on a T-maze showed significant increases in *eIF4A3* mRNA expression in the dorsomedial (DM) and dorsolateral (DL) striatum compared to caged control (CC) rats ($n = 11$ per group). *Significantly different from CC ($p = 0.0001$). (B–G) Degree of correlation between behavioral performance (trials to criterion) on the response-reversal task and *eIF4A3* mRNA expression (average gray value from densitometric analysis) in DM striatum (A), DL striatum (B), CA1 of dorsal hippocampus (C), CA3 of dorsal hippocampus (D), upper blade of dentate gyrus (DGub; E), and lower blade of dentate gyrus (DGlb; F). *Significant correlation ($p < 0.05$).

field area with eIF4A3 protein increased relatively rapidly in response to exploration of a novel environment, this timeframe is not unrealistic for neuronal protein translation and would thus supply an alternative interpretation of the presently observed changes in eIF4A3 protein expression.

Our novel findings for *eIF4A3* mRNA and protein levels parallel those previously reported for *Arc* mRNA (Guzowski et al., 1999; Chawla et al., 2005; Daberkow et al., 2007). First, *eIF4A3* mRNA is rapidly increased in the dorsal striatum (Daberkow et al., 2007), as well as in CA1, CA3, and DGub of the dorsal hippocampus (Guzowski et al., 1999) of adult rats engaged in brief (5 min) exploration of a novel environment. Second, like *Arc* mRNA (Chawla et al., 2005; Vazdarjanova et al., 2006), *eIF4A3* mRNA expression was not increased in DGlb. Third, there is a significant correlation between *eIF4A3* mRNA levels in the DM striatum, but not DL striatum or dorsal hippocampal subfields, and trials to criterion on a striatally-based, response-reversal learning task (Daberkow et al., 2007, 2008). Fourth, *Arc* mRNA colocalized with eIF4A3 protein *in vivo* following striatally-based learning, as it has been shown to do in the hippocampus *in vitro* as well (Giorgi et al., 2007). Finally, there was a significant activity-dependent increase in eIF4A3 protein distribution that returned to basal levels 60 min after exploration of a novel environment, in close parallel to the time frame of *Arc* transcription, trafficking, and protein translation (Vazdarjanova et al., 2006; Baez et al., 2011). These findings suggest that *eIF4A3* may critically regulate *Arc*-dependent synaptic plasticity. In this regard, it is interesting that EJC components also regulate mitogen-activated protein kinase (MAPK) splicing in *Drosophila* (Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010). MAPK is critical for ERK phosphorylation, and thus, *Arc* mRNA targeting to activated synapses (Huang et al., 2007; Wang et al., 2009). The *in vivo* observations reported herein are the first to demonstrate activity-related regulation of the core EJC component *eIF4A3*, with the expression of *eIF4A3* mRNA showing notable similarities to the behaviorally-induced expression of *Arc* mRNA previously reported by others (Guzowski et al., 1999; Chawla et al., 2005; Vazdarjanova et al., 2006) and us (Daberkow et al., 2007, 2008; Pastuzyn et al., 2012). The extent to which eIF4A3 is necessary for *Arc*-dependent neuroplasticity in the brain *in vivo* is currently under further investigation.

Presently, the basis for the activity-related expression of *eIF4A3* mRNA levels is unknown, but the current findings further highlight that neurons dynamically regulate mRNAs involved in post-transcriptional processing, such as RNA binding. For example, the mediator of cap-binding activity during protein translation initiation, *eIF4E* mRNA, shows dendritic localization and increased association with PSD-95 upon KCl stimulation *in vitro* (Moon et al., 2009), demonstrating that neurons not only dendritically localize effector genes, but also the factors that facilitate local translation of those effector genes. Additionally, the brain-specific, embryonic lethal, abnormal vision protein (ELAV)/Hu family of RNA-binding proteins, which are known to contribute to growth-associated protein 43 (*GAP-43*) mRNA localization and cytoplasmic stabilization, also show dendritic localization (Bolognani et al., 2004) and activity-dependent expression following spatial learning (Pascale et al., 2004). Furthermore, ELAV/Hu expression is modulated by glutamatergic (seizures) or dopaminergic (cocaine) signaling (Tiruchinapalli et al., 2008), thereby demonstrating a role of classical neurotransmitter systems converging onto activity-dependent, post-transcriptional regulatory elements to modulate neuronal responses. Thus, future work will clarify whether neuronal stimulation results in new transcriptional activation of *eIF4A3* or alternative post-transcriptional processing of *eIF4A3*, so as to determine the basis for the observed activity-dependent changes in *eIF4A3* mRNA and protein levels.

CONCLUSIONS

Our present findings reveal activity-related increases in *eIF4A3* mRNA and protein distribution in the adult mammalian CNS *in vivo*. Previous reports indicate a unique function for eIF4A3 in regulating expression of *Arc* mRNA (Giorgi et al., 2007), a key mediator of synaptic plasticity (Guzowski et al., 2000; Chowdhury et al., 2006; Rial Verde et al., 2006) and basal ganglia-mediated learning consolidation (Pastuzyn et al., 2012). Herein, we demonstrate that *eIF4A3*, but not *Magoh*, mRNA shows striking similarities to *Arc* mRNA in terms of patterns of expression in the context of spatial exploration (Guzowski et al., 1999; Chawla et al., 2005) and the correlation of mRNA expression with learning (Daberkow et al., 2007, 2008), implicating eIF4A3 as a potential regulator of *Arc* expression *in vivo*. These present observations suggest that neurons coordinate the expression of not only effector genes, such as *Arc*, but also post-transcriptional regulatory factors and pathways required by those specific effector genes. Dysfunction in these factors and pathways potentially disrupts neuroplasticity processes and can lead to neurological disorders (Dahm and Macchi, 2007; Tarpey et al., 2007). However, how these regulatory elements are themselves expressed and behaviorally activated in the adult, mammalian brain is currently less clear. Future studies are thus needed to determine the precise mechanisms by which *eIF4A3* is regulated *in vivo*, as well as the functional roles that *eIF4A3* may be playing in the post-transcriptional regulation of select effector genes within activated neurons. Such knowledge will afford a more complete understanding of both normal and abnormal neuroplasticity processes required of the adult mammalian CNS.

CONFLICT OF INTEREST

None.

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CHAPTER 3

DIFFERENCES IN ACTIVITY-INDUCED CYTOPLASMIC MRNA LOCALIZATION IN STRIATAL EFFERENT NEURONS AND DISRUPTION BY METHAMPHETAMINE

Abstract

Striatal efferent neurons differ on many levels: neuroanatomical projections, neurotransmitter receptors, and neuropeptide expression. We have previously reported that following novel environment exploration, more striatonigral than striatopallidal neurons have cytoplasmic *Arc* mRNA despite equivalent induction of the *Arc* gene and that such localization is disrupted subsequent to partial monoamine loss induced by methamphetamine. Thus, to assess whether these phenotypic localization differences reflect intrinsic posttranscriptional processing differences between striatal efferent neurons, or differences unique to rapidly induced, dendritically localized *Arc*, we examined the *in vivo* expression of the similarly activated mRNA, *zif268/egr-1* in normal animals and animals with methamphetamine-induced neurotoxicity. As a control mRNA, we examined the expression of constitutively expressed *Map2* (microtubule-associated protein 2). Similar to *Arc*, more striatonigral neurons had *zif268* in the peri-nuclear cytoplasm after novel environment exploration. However, closer examination of *zif268* mRNA distribution 30 minutes after novelty revealed that more striatopallidal neurons had *zif268* in the nucleus. Furthermore,

partial monoamine loss disrupted activity-induced cytoplasmic *zif268* localization in both neuron subpopulations. Conversely, there were no significant phenotypic differences in *Map2* subcellular localization at any time point. These present data suggest that striatonigral vs. striatopallidal neurons may utilize differential activity-dependent regulation of nuclear posttranscriptional mRNA processing and/or nuclear mRNA export *in vivo*.

Introduction

The basal ganglia critically mediate normal motor behavior and action selection by linking cortical activation to the output systems necessary to execute such intended behavior (DeLong and Wichmann, 2009), with the striatum being the key input nucleus regulating such behaviors. The dorsal striatum is involved in reward and goal-directed behaviors (Graybiel, 1995; DeCoteau and Kesner, 2000; Balleine et al., 2007), and synaptic plasticity within this brain region is critical to the maintenance of such learned behaviors (Calabresi et al., 2007; Surmeier et al., 2009). Approximately 95% of neurons in striatum are medium spiny neurons, and these efferent neurons can be divided into two subpopulations: striatonigral "direct" pathway neurons and striatopallidal "indirect" pathway neurons (Grillner et al., 2005; DeLong and Wichmann, 2009). While much of our understanding of synaptic plasticity processes in general have been elucidated within hippocampal and cortical systems (*c.f.* (Bramham et al., 2008; Shepherd and Bear, 2011)), the heterogeneous distribution of spiny efferent neurons within striatum has slowed our understanding of these essential cellular processes within this brain structure *in vivo*. Therefore, examining the precise cellular and molecular processes mediating such striatal neuroplasticity is crucial to understanding both normal, as well as aberrant, functions of the basal ganglia so as to

identify novel therapeutic targets and direct treatment strategies underlying basal ganglia-mediated pathologies.

The immediate-early gene, *Arc/Arg3.1* (activity-regulated, cytoskeleton associated protein) is heavily implicated in learning and memory processes (Guzowski et al., 2001; Messaoudi et al., 2007), including those mediated by the basal ganglia (Hearing et al., 2011; Pastuzyn et al., 2012). We have routinely demonstrated that following spatial exploration of a novel environment by normal rats, more striatonigral than striatopallidal neurons contain cytoplasmic *Arc* mRNA expression, despite equivalent numbers of the two neuron populations showing transcriptional activation of the *Arc* gene (Daberkow et al., 2007; Barker-Haliski et al., 2012b). Furthermore, in the context of partial monoamine loss induced by methamphetamine (METH), such phenotypic differences are lost both in response to novelty (Barker-Haliski et al., 2012b) and to response-reversal learning on a striatally mediated task (Daberkow et al., 2008). Thus, striatal efferent neurons either differentially regulate cytoplasmic *Arc* mRNA localization or possess intrinsic differences in nuclear post-transcriptional processing and/or mRNA export dynamics that have, heretofore, remained relatively unexplored *in vivo*. Thus, to begin to understand the phenotypic differences in nuclear posttranscriptional processing and mRNA export within striatal efferent neurons, we investigated the expression of candidate mRNAs that share discrete expression and trafficking characteristics with *Arc* mRNA. To this end, we examined the subcellular localization of these mRNAs following spatial exploration of a novel environment, a paradigm routinely used to examine subcellular distribution of *Arc* mRNA (Guzowski et al., 1999; Daberkow et al., 2007; Barker-Haliski et al., 2012b). Furthermore, we selectively disrupted normal striatal function by using a partial monoamine depletion induced by the psychostimulant, methamphetamine (METH), so as to more closely examine how

dopaminergic tone may regulate subcellular mRNA distribution within striatal efferent neurons.

Like *Arc*, *zif268* mRNA is subject to a similar time-course of transcriptional regulation (Richardson et al., 1992; Guzowski et al., 1999; Guzowski et al., 2001; Saha et al., 2011), can be induced by NMDA receptor activation in striatum (Gass et al., 1993), and is heavily implicated in long-term synaptic plasticity (Jones et al., 2001), including striatal learning and memory processes (Valjent et al., 2006). Unlike *Arc*, however (Steward et al., 1998), *zif268* is not trafficked to dendrites for local protein translation, but rather encodes a transcription factor essential to the induction of other neuronal mRNAs (Davis et al., 2003; Knapska and Kaczmarek, 2004). Additionally, *Arc* and *zif268* mRNAs differ at the level of cytoplasmic mRNA regulation. *Arc* mRNA is subject to cytoplasmic mRNA decay mediated by the exon-junction complex component, eIF4A3 (Giorgi et al., 2007), and we have recently demonstrated that eIF4A3 colocalizes with *Arc* in striatum *in vivo* (Barker-Haliski et al., 2012a). However, *zif268* mRNA is not subject to such translation-dependent decay processes (TDD (Giorgi et al., 2007)) that could contribute to differences in cytoplasmic mRNA levels within striatal efferent neurons. Because *zif268* is subject to similar transcriptional regulation as *Arc*, but is not subject to cytoplasmic TDD, it is an effective tool to explore the possibility that there are differences in posttranscriptional mRNA regulation within striatal efferent neurons in both normal states and in the context of partial monoamine depletion.

We also examined *Map2* mRNA expression within identified striatal efferent neurons, because of the subcellular distribution profile *Map2* shares with *Arc*, as well as the role of Map2 protein in mediating normal and aberrant neuroplasticity within striatum. *Map2* is a constitutively expressed, dendritically localized mRNA that is subject to activity-

dependent protein translation (Garner et al., 1988; Blichenberg et al., 1999; Steward and Halpain, 1999). Importantly for this present study, *Map2* mRNA associates with eIF4A3 and the EJC, but is not subject to cytoplasmic regulation by TDD (Giorgi et al., 2007), thus also differentiating it from *Arc* mRNA in the possible contribution of TDD to cytoplasmic mRNA expression differences within striatal efferent neurons. Map2 protein plays a critical role regulating cytoskeletal architecture in response to synaptic activity, and Map2 dysfunction has been implicated in several neurological and neurodegenerative diseases (Benitez-King et al., 2004). Additionally, Arc protein interacts with Map2 protein in dendrites in response to synaptic activation (Fujimoto et al., 2004). Interestingly, both a neurotoxic regimen of METH (Zhang et al., 2007) or selective serotonin depletion (Whitaker-Azmitia et al., 1995) reduces Map2 immunoreactivity in the rat brain, and mutant mice that selectively lose nigrostriatal dopamine show deficits in striatal *Map2* gene expression (Sola et al., 1993). Therefore, *Map2* differs from *Arc* mRNA at the level of transcriptional activation, but interacts with Arc protein to mediate normal synaptic plasticity processes. Also, much like *Arc*, *Map2* mRNA expression in rat striatum can be severely disrupted by monoamine depletion. Yet, whether there are phenotypic differences in the expression and activity-dependent subcellular localization of *Map2* mRNA within striatal efferent neuron subpopulations of normal animals is currently unknown and was thus a goal of the present study.

The differences between *Arc* and both *zif268* and *Map2* mRNAs presently investigated in this study offer an intriguing means to begin to examine activity-dependent, posttranscriptional mRNA nuclear export dynamics in striatal efferent neurons. Determining the phenotypic subcellular localization of these mRNAs in normal animals thus attempted to clarify whether cytoplasmic expression differences previously observed for *Arc* mRNA arise

as a consequence of neuronal phenotype-specific differences in mRNA regulation or whether such differences present a further unique characteristic of striatal *Arc* mRNA expression. Thus, the goal of the present studies was to apply targeted properties of *Arc* mRNA activation and cytoplasmic localization to selectively examine candidate mRNAs that share such lifecycle characteristics with *Arc* so as to tease apart the precise means by which phenotypic expression differences in cytoplasmic *Arc* mRNA localization arise within striatal efferent neuron subpopulations (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b). Additionally, we disrupted normal dopaminergic input into the striatum using a neurotoxic regimen of METH so as to more clearly determine the potential role of dopamine in mediating such normal subcellular processing. These studies were carried out to ultimately address the precise subcellular processing differences present within striatal efferent neurons so as to further understand both normal and aberrant neuroplasticity processes within the key input nucleus of the basal ganglia.

Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC; 275-300g) were singly housed in tub cages in a room controlled for temperature and lighting (12:12 hr). All animal care and experimental procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (8th Ed.), followed the ARRIVE guidelines (Kilkenny et al., 2010), and were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Methamphetamine Treatment

Rats were treated with a neurotoxic regimen of (±)-methamphetamine hydrochloride (NIDA, Research Triangle Park, NC) so as to induce long-term, partial monoamine depletion in dorsal striatum, as previously described (Daberkow et al., 2008; Son et al., 2011; Barker-Haliski et al., 2012b). On the METH treatment day, rats were housed 5-6 animals per cage. Rats received four injections of either (±)-METH (10 mg/kg free base, s.c.) or 0.9% saline (1 mL/kg, s.c.) at 2-hr intervals, with rectal temperatures recorded hourly. If core temperature exceeded 41°C, the rat was removed and placed in a separate cage over ice to decrease hyperthermia. Twelve hours after the final injection, rats were returned to home cages and given free access to food and water until behavioral manipulations three weeks later.

Behavioral Activation via Novel Environment Exploration

Three weeks after saline- or METH-pretreatment, rats were divided into three experimental groups (caged controls (CC), "5-min", "30-min"), as previously described (Guzowski et al., 1999; Daberkow et al., 2007; Barker-Haliski et al., 2012b). Each group consisted of 8-9 rats / time point (saline-pretreated) or 5-12 rats / time point (METH-pretreated), Animals were sacrificed by CO_2 exposure, decapitated, and brains immediately removed and flash-frozen in 2-methylbutane (Mallinckrodt Baker, Phillipsburg, NJ) chilled on dry ice.

Upon observing phenotypic differences in cytoplasmic expression of *zif268* mRNA, we generated a second cohort of animals with longer time points between novel environment exploration and sacrifice, so as to investigate the expression of *Map2* mRNA in

normal animals. Rats in this cohort were divided into 5 experimental groups (CC, "5-min", "30-min", “60-min”, “90-min”), each consisting of 4 rats / time point.

Tissue Preparation

Twelve-μm cryosections of striatum (Cambridge Instruments, Bayreuth, Germany; Bregma: +1.60 to -0.8 mm) (Paxinos and Watson, 1998) were thaw-mounted onto SuperFrost Plus slides (VWR, Batavia, IL), then postfixed as previously described (Ganguly and Keefe, 2001).

DAT Autoradiography

METH-induced DA depletions in striatum were analyzed using [I^{125}]RTI-55 binding to the dopamine transporter (DAT), as described previously (Boja et al., 1992; Pastuzyn et al., 2012). DAT binding levels were normalized to percent of saline controls.

Dual Fluorescent *In Situ* Hybridization Histochemistry

Expression of *zif268* mRNA in striatal efferent neurons was determined by double-label fluorescence *in situ* hybridization histochemistry (FISH) for *zif268 or Map2* and *ppe* mRNAs, as previously described for *Arc* (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b; Barker-Haliski et al., 2012a; Howard et al., 2013). Full-length ribonucleotide probes complementary to mRNAs for *zif268* (Milbrandt, 1987) and *ppe* (Yoshikawa et al., 1984) were synthesized from cDNAs using digoxigenin-UTP (DIG-UTP) and fluorescein-UTP (FITC-UTP) with T7 and SP6 RNA polymerases and DIG- and FITC-UTP RNA-labeling kits, respectively (Roche Applied Science, Indianapolis, IN). A full-length rat *Map2* cDNA-containing vector was purchased for plasmid isolation ([GenBank: BC081835]; Open

Biosystems, Huntsville, AL), cDNA linearized (*EcoRI*; Roche Applied Science, Indianapolis, IN), and probe transcribed with DIG-UTP and T7 RNA polymerase (Roche Applied Science, Indianapolis, IN). Ribonucleotide probes were hybridized and detected as previously described (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b; Barker-Haliski et al., 2012a).

Image Acquisition

For *zif268/ppe* or *Map2/ppe* dual *in situ* hybridization histochemistry, a 0.18 mm^2 montage from dorsomedial (DM) striatum was captured with an FV1000 confocal laser-scanning microscope (Olympus) with motorized stage (Prior Scientific) using a 60x, 1.45 NA oil-immersion lens (plan APO) and 488-nm Ar and 543-nm and 633-nm HeNe lasers. Areas of analysis were *z*-sectioned in 1-μm-thick optical sections (10 *z*-sections per image; (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b). The numbers of *ppe*-pos and *ppe*-neg neurons with *zif268* or *Map2* mRNA staining as 1-2 foci of intranuclear staining ("foci") or labeling in the cytoplasm only ("cytoplasmic") were determined (Guzowski et al., 1999; Daberkow et al., 2007, 2008). Additionally, we generated two new dependent measures to fully capture the nature of the subcellular distribution of the mRNA signals. Cells with diffuse mRNA expression throughout the nucleus only across 2 or more *z*-sections were counted as "full nucleus", whereas cells simultaneously containing diffuse nuclear and cytoplasmic labeling across 2 or more consecutive *z*-sections were labeled "whole cell". The phenotypic identity of striatal efferent neurons (*ppe* +/-) was withheld from image analysis until after making subcellular *zif268* and *Map2* expression assignments. As previously justified (Daberkow et al., 2007), phenotypic *ppe* expression was implemented to differentiate striatal efferent neuron subtypes because approximately 95% of neurons within striatum are

spiny efferent neurons (Gerfen and Young, 1988; Le Moine and Bloch, 1995). Furthermore, previous work by Vazdarjanova and colleagues demonstrated that *Arc* mRNA is only expressed in GAD-67 positive striatal neurons (Vazdarjanova et al., 2006), with GAD-67 only expressed in striatal efferent neurons and a small subpopulation of striatal interneurons that comprises ~1% of the entire striatal neuron population (Mercugliano et al., 1992), thus making *ppe* an effective phenotypic marker to differentiate striatonigral (*ppe*-neg) and striatopallidal (*ppe*-pos) neurons (Daberkow et al., 2007).

Statistical Analysis

Analysis of DAT binding was accomplished by two-sample *t*-tests. Expression of mRNA in each subcellular compartment in normal and METH-pretreated rats was compared across neuronal phenotype by MANOVA (time, phenotype). *Post-hoc* analyses of significant interactions were achieved via Tukey-Kramer test for between-subjects factors (time) or *post-hoc t*-tests for the within-subjects factor (phenotype). For all analyses, *p*-values <0.05 were considered to be significant.

Results

In Normal Animals, More Striatonigral Neurons Have Cytoplasmic Localization of *zif268* after Novel Environment Exploration

We have previously investigated the subcellular distribution of *Arc* mRNA within identified striatal efferent neurons in response to novel environment exploration (Daberkow et al., 2007; Barker-Haliski et al., 2012b), and previous reports by others have demonstrated a similar time course of activation for *zif268* mRNA within the hippocampus using such a novel environment exploration paradigm (Guzowski et al., 2001). So, we presently employed

this novel environment exploration paradigm to examine *zif268* transcriptional activation and cytoplasmic localization within identified striatal efferent neurons in saline-pretreated rats (Figure 3.1). Exposure of saline-pretreated rats to a novel environment resulted in a trend to induce transcriptional activation of *zif268* above baseline expression levels (Figure 3.1B; $F_{(2,21)}=3.04$, $p=0.07$). However, there was no effect of phenotype ($F_{(1,21)}=0.33$, $p>0.5$) or phenotype x time interaction ($F_{(2,21)}=0.25$, $p>0.7$) in the numbers of cells with intranuclear *zif268* foci.

We observed a significant time-dependent increase in cytoplasmic localization of *zif268* mRNA in response to novel environment exploration (Figure 3.1C; $F_{(2,21)}=4.27$, $p=0.03$). Furthermore, like *Arc* mRNA (Daberkow et al., 2007; Barker-Haliski et al., 2012b), there was a significant effect of phenotype on numbers of cells with *zif268* in the cytoplasm ($F_{(1,21)}=10.85$, $p=0.004$), with more *ppe*-neg neurons having cytoplasmic *zif268* than *ppe*-pos striatopallidal neurons. There was no significant time x phenotype interaction ($F_{(2,21)}=0.91$, $p>0.4$). *Post-hoc* analysis of main effect of time revealed that there was an increase in the numbers of neurons with cytoplasmic *zif268* mRNA in the 30-min group relative to the CC group ($p=0.02$), but not relative to the 5-min group ($p=0.16$). Thus, spatial exploration of a novel environment induced a robust subcellular distribution of *zif268* mRNA, with more *ppe*-neg neurons displaying cytoplasmic *zif268* expression than *ppe*-pos neurons.

In Normal Animals, More Striatopallidal Neurons Have *zif268* in the Nucleus 30 Minutes after Novel Environment Exploration

The divergent numbers of *ppe*-neg neurons with cytoplasmic *zif268* in normal animals is intriguing, particularly in relation to our previous observations that greater numbers of *ppe-neg* cells also have cytoplasmic *Arc* mRNA expression (Daberkow et al., 2007; Barker-Haliski

et al., 2012b). Importantly, unlike *Arc*, *zif268* mRNA is not subject to regulation by cytoplasmic TDD (Giorgi et al., 2007); therefore, the present data with *zif268* suggest that differences in cytoplasmic mRNA stability between striatal efferent neuron subpopulations are not likely contributing to the greater number of *ppe*-neg cells with cytoplasmic *zif268* and *Arc* mRNA expression. Thus, we examined more closely the subcellular distribution of *zif268* mRNA in striatonigral efferent neurons by performing a subsequent, more stringent analysis of mRNA distribution within striatal efferent neurons within the 30-min group only.

For this analysis, cells were classified as having "full nucleus", "cytoplasmic-only", or "whole cell" labeling (Figure 3.2A; see Methods). When numbers of cells with "full nucleus" staining for *zif268* were analyzed, there was a significant effect of phenotype ($t=-2.51$, $p=0.04$), with neurons with full nuclei being approximately 2:1 *ppe*-pos (Figure 3.2B). However, when this more stringent analysis was used to assess cells with *zif268* throughout the cell ("whole cell" labeling), there was a significant effect of phenotype in the opposite direction, such that there were more *ppe*-neg neurons positive for "whole cell" labeling of *zif268* (Figure 3.2B; $t=3.07$, $p<0.02$). This analysis also confirmed our prior observations for cells positive for cytoplasmic *zif268* in the 30-min group, with more *ppe-neg* neurons containing cytoplasmic *zif268* than *ppe-pos* (Figure 3.2B; $t=6.14$, $p<0.001$). Thus, a more stringent analysis of the subcellular distribution of activity-induced mRNAs revealed a profound difference in cytoplasmic distribution dynamics between striatal efferent neuron subpopulations.

Constitutively Expressed *Map2* Shows No Phenotype-specific Cytoplasmic Expression Differences in Normal Animals

Upon observing the differential cytoplasmic expression of *zif268* in striatal efferent neurons of saline-pretreated rats, we then investigated the subcellular expression of *Map2*, a constitutively expressed mRNA that is subject to activity-dependent dendritic transport and protein synthesis (Torre and Steward, 1992; Steward and Halpain, 1999). Because Steward and colleagues demonstrated time-dependent fluctuations in Map2 protein immunoreactivity in the dentate gyrus of normal animals (Steward and Halpain, 1999), additional time points were examined to assess cell body-level *Map2* mRNA localization at similar periods after neuronal activity (Figure 3.3A). We initially examined signal intensity (mean gray value) and percent field area with pixels positive for *Map2* signal to determine whether there was any detectable change in *Map2* localization following novel environment exploration (Figure 3.3B and 3.3C). Such densitometric analysis revealed a significant time-dependent change in signal intensity (Figure 3.3B; $F_{(4,18)}=3.78,\ p<0.03$) and a strong trend for an activity-dependent change in percent field area (Figure 3.3C; $F_{(4,18)}=2.92,\ p=0.06$). *Post-hoc* analysis of mean gray values revealed that the 5-min group differed significantly from the CC ($p<0.03$) and 30-min ($p=0.03$) groups, with a strong trend to differ from the 60-min ($p<0.06$) and 90-min ($p<0.08$) groups as well. Thus, densitometric analysis of *Map2* mRNA signal within dorsal striatum followed a similar time course of mRNA distribution that was previously reported for changes in Map2 protein immunoreactivity in hippocampus (Steward and Halpain, 1999).

To determine whether *Map2* mRNA also had differential cytoplasmic localization in striatal efferent neurons in response to behavioral activation in normal animals, we counted *Map2* positive cells, as performed above for *zif268* and as previously for *Arc* mRNA

(Guzowski et al., 1999; Daberkow et al., 2007; Barker-Haliski et al., 2012b). *Map2* nuclear foci were not detected in medium spiny neurons (data not shown), as cells predominately displayed either cytoplasmic or whole cell labeling (Figure 3.3A). Analysis of the numbers of cells with cytoplasmic-only *Map2* labeling revealed no significant main effect of time (Figure 3.3D; $F_{(4,14)}=1.38,\ p>0.2$), no significant effect of phenotype ($F_{(4,14)}=0.83,\ p>0.3$), and no significant phenotype x time interaction ($F_{(4,14)}=1.25,\ p>0.3$). However, analysis of the numbers of cells with "whole cell" labeling of *Map2* mRNA expression showed a significant main effect of time (Figure 3.3E; $F_{(4,14)}=3.64,\ p=0.03$), with *post-hoc* analysis revealing that the 5-min group was significantly different from all other groups ($p<0.05$). There was no significant effect of phenotype on the numbers of cells with "whole cell" labeling for *Map2* ($F_{(1,14)}=1.63,\ p>0.2$) and no significant phenotype x time interaction ($F_{(4,14)}=0.26,\ p=0.9$). Thus, subcellular distribution of the constitutively expressed, non-IEG *Map2* was not different between striatonigral and striatopallidal neurons. These data suggest that the phenotypic differences in *Arc* (Daberkow et al., 2007; Barker-Haliski et al., 2012b) and *zif268* mRNA localization in striatonigral *vs.* striatopallidal neurons of normal animals may reflect differential posttranscriptional processing and nuclear export of rapidly induced mRNAs.

METH-Induced Partial Monoamine Depletion Disrupts Cytoplasmic Localization of *zif268* mRNA

Given that the data above suggested the possibility of differential regulation of post-transcriptional processing and nuclear export of rapidly induced mRNAs in striatal efferent neurons, and given the differential effects of DA on these two populations of neurons (Gerfen and Surmeier, 2011), we next investigated how disruption of normal dopaminergic input onto striatal efferent neurons affected *zif268* subcellular distribution, as previous

reports have demonstrated significant impairments in striatal IEG expression after METH-induced neurotoxicity (Horner and Keefe, 2006; Daberkow et al., 2008; Belcher et al., 2009; Barker-Haliski et al., 2012b). A neurotoxic regimen of METH decreased DAT binding to 52.6±4.1% (*t=8.32, p<0.0001*) of control levels in DM striatum and to 67.8±3.6% (*t=6.46, p<0.0001*) of control levels in dorsolateral (DL) striatum (Figure 3.4A; (Chapman et al., 2001; Daberkow et al., 2008; Barker-Haliski et al., 2012b)).

Analysis of the numbers of cells with intranuclear foci of *zif268* mRNA expression in rats with METH-induced DA loss that explored a novel environment for 5 minutes revealed a slight trend toward a main effect of time (Figure 3.4B; $F_{(1,19)}=2.11, p=0.15$), as was seen in saline-pretreated rats (Figure 3.1B). As was also the case for the saline-pretreated rats, there was no significant effect of neuronal phenotype ($F_{(1,19)}=0.18, p>0.6$) and no significant phenotype x time interaction ($F_{(2,19)}=1.19, p>0.3$).

Analysis of the numbers of cells with cytoplasmic-only expression of *zif268* mRNA expression in rats with METH-induced DA loss that explored a novel environment for 5 minutes revealed an interesting divergence from saline-pretreated rats. There was a significant effect of time ($F_{(2,19)}=5.13, p<0.02$), a significant main effect of phenotype ($F_{(1,19)}=9.06, p<0.008$), and a significant phenotype x time interaction (Figure 3.4C; $F_{(2,19)}=5.68, p<0.02$). *Post-hoc* analysis of the significant interaction revealed that activity-dependent increases in cytoplasmic *zif268* mRNA localization were disrupted by partial monoamine depletion. Although all groups had more *ppe*-neg neurons with *zif268* mRNA in the cytoplasm, this difference was only significant in the CC group (*t=4.93, p<0.001*). Furthermore, only *ppe*-neg neurons had a time-dependent change in cytoplasmic *zif268* localization (*ppe*-pos*: $F_{(2,19)}=1.47, p>0.2$; ppe*-neg*: $F_{(2,19)}=6.84, p<0.01$*); however, unlike the case in saline-pretreated rats, in these METH-pretreated rats, there were more *ppe-neg*

neurons with *zif268* in the cytoplasm in the CC group than in the 5- and 30-min groups (Figure 3.3C; 5-min, $p<0.03$; 30-min, $p<0.02$). Interestingly, the numbers of cells positive for *zif268* expression in the cytoplasm of METH-pretreated CC was not different from the numbers of neurons with *zif268* mRNA expression in the cytoplasm of the normal CC rats (*ppe*-pos: $t=-1.41$, $p>0.1$; *ppe*-neg: $t=-0.48$, $p>0.6$; compare Figures 3.1C and 3.4C). Thus, partial DA depletion significantly impairs activity-dependent cytoplasmic localization of *zif268* in *ppe*-pos and *ppe*-neg neurons, as well as ablating the phenotypic expression difference in cytoplasmic *zif268* mRNA localization 30 minutes after novel environment exploration (Figure 3.4D). These data thus suggest that DA may normally critically mediate nuclear mRNA export within striatal efferent neurons, as partial DA depletion disrupts activity-induced cytoplasmic localization of *zif268* mRNA, in line with our previous observations for *Arc* mRNA under similar circumstances (Daberkow et al., 2007; Barker-Haliski et al., 2012b).

Discussion

We presently report differential subcellular distribution of the IEG *zif268* mRNA, but not of the constitutively expressed, non-IEG MAP2 mRNA, in striatonigral *vs.* striatopallidal neurons of rats subsequent to exploration of a novel environment. While our results for transcriptional activation of *zif268* were less robust than previous reports for *zif268* expression in the hippocampus (Guzowski et al., 2001), other reports indicate significant basal expression of *zif268* (Worley et al., 1991; Roberts et al., 1996; Zangenehpour and Chaudhuri, 2002; Knapska and Kaczmarek, 2004), which likely decreased our ability to detect the *de novo* transcription occurring in response to novelty-induced exploration. Importantly, our findings that more striatonigral neurons contain cytoplasmic *zif268*

expression relative to striatopallidal neurons despite equivalent mRNA transcription mirrors our previous reports of the differential cytoplasmic localization of *Arc* within striatal efferent neurons (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b). Furthermore, our additional analysis of the discrete subcellular distribution of *zif268* mRNA 30 minutes after novel environment exploration revealed that more striatopallidal nuclei were diffusely filled with *zif268* mRNA signal relative to striatonigral neurons. Interestingly, *Map2* did not show such differences in activity-dependent cytoplasmic distribution within striatal efferent neurons subpopulations, suggesting that activity-induced distribution of IEGs *per se* from the nucleus to the cytoplasm is different in striatopallidal and striatonigral neurons. Additionally, in the context of partial DA depletion, normal activity-dependent cytoplasmic localization of *zif268* is disrupted, as previously observed for *Arc* (Daberkow et al., 2008; Barker-Haliski et al., 2012b). Thus, these data further suggest that there may be differential post-transcriptional mRNA processing or nucleocytoplasmic transport of IEGs in striatonigral *vs.* striatopallidal neurons *in vivo*, and that DA may participate in regulating such functions. Our present observations thus demonstrate a novel difference between distinct neuronal subtypes in the same brain region at the level of nuclear mRNA regulation or nucleocytoplasmic transport and further emphasize fundamental differences in neuroplasticity processes within these two efferent neuron populations of the striatum.

It is well established that *zif268* plays a critical role meditating normal LTP processes *in vivo*, thus providing functional significance for the observed changes in subcellular localization of *zif268* mRNA within striatal efferent neuron subpopulations in response to exploration of a novel environment. First, tetanic stimulation to induce LTP in hippocampal slice cultures robustly upregulates of *zif268* expression (Mackler et al., 1992; Roberts et al., 1996). Second, *zif268* upregulation specifically correlates with the persistence of LTP

(Richardson et al., 1992; Abraham et al., 1993). Finally, *zif268* mutant mice show no changes in the induction of and early phases of LTP within the dentate gyrus, but maintenance of late LTP in these animals is disrupted, thereby demonstrating the critical role *zif268* plays in stabilizing late LTP (Jones et al., 2001). Thus in normal animals, it is likely that spatial exploration of a novel environment is associated with activation *zif268* transcription and cytoplasmic distribution in striatal efferent neurons so as to mediate neuroplasticity essential to learning arising in the context of such novelty exposure.

We presently demonstrate that striatal efferent neuron subpopulations differentially regulate the cytoplasmic localization of activity-regulated *zif268*, similar to our previous observations for *Arc* mRNA expression and subcellular distribution (Daberkow et al., 2007; Barker-Haliski et al., 2012b). However, unlike *Arc, zif268* is not a substrate for cytoplasmic TDD (Giorgi et al., 2007), which could have been a basis for diminished detectable levels of cytoplasmic mRNA within striatopallidal vs. striatonigral neurons, and thereby contribute to the observed phenotypic differences. Furthermore, the present results also show that whereas more striatonigral neurons have *zif268* mRNA expression in the cytoplasm, more striatopallidal neurons have nuclei full of *zif268* mRNA. We then asked whether such subcellular differences were also present for *Arc* mRNA. Conducting this same analysis for "full" and "whole cell" labeling on existing 30-min group *Arc/ppe* images shown to have more striatonigral than striatopallidal neurons with *Arc* mRNA in the cytoplasm (Barker-Haliski et al., 2012b) demonstrated that more striatopallidal cells contained diffuse, whole cell labeling for *Arc* mRNA ($t=-4.48$, $p=0.001$; data not shown), but no phenotype-specific difference in numbers of cells with full nuclei ($t=1.73$, $p>0.1$; data not shown). Altogether, these observations suggest that there are differences between striatonigral and striatopallidal neurons with respect to posttranscriptional processing in the nucleus or nucleocytoplasmic

transport of these IEG mRNAs. How nuclear export proceeds in neurons in general, and in striatal efferent neurons in particular, thus requires further scrutiny. While it is known that *Arc* forms an mRNA-protein complex that contributes to nuclear export and cytoplasmic trafficking (Le Hir et al., 2001; Kanai et al., 2004; Giorgi et al., 2007), whether such post-transcriptional processing occurs for the regulation of *zif268* mRNA is currently unknown.

The fact that there is differential regulation of *zif268* mRNA localization in striatonigral *vs.* striatopallidal neurons is not immediately surprising given the opposing functionality of these neuronal subpopulations and known cellular differences (DeLong and Wichmann, 2009). Striatal efferent neuron subpopulations selectively express DA and adenosine receptors (Gerfen et al., 1990; Ferre and Fuxe, 1992; Surmeier et al., 1992; Tozzi et al., 2007), thus leading to neuron-specific differential activation of intracellular signaling cascades, such as PKA, which could contribute to the observed phenotypic differences in the subcellular localization of *zif268* (present data) and *Arc* (Daberkow et al., 2007; Barker-Haliski et al., 2012b) mRNAs. The data presented herein from rats with METH-induced monoamine loss support the possibility DA signaling may in fact play a role in the differential processes. Prior work from our lab has established that METH-induced DA loss is associated with disruption of phasic DA neurotransmission (Howard et al., 2011), and recent modeling work suggests that D1 dopamine receptors may be preferentially affected by changes in phasic DA signaling (Dreyer et al., 2010). Interestingly, in the present study, the basal numbers of cells with cytoplasmic *zif268* mRNA localization (i.e., those in the CC group) were not significantly different between saline- and METH-pretreated rats (Figures 3.1C and 3.4C). However, the normal increase in the numbers of striatonigral neurons, which selectively express the D1 subtype of DA receptors (Gerfen and Surmeier, 2011), with *zif268* mRNA in the cytoplasm induced by spatial exploration of a novel environment, was

abolished in METH-pretreated rats. Interestingly, we have previously demonstrated similar disruption of cytoplasmic *Arc* mRNA localization in striatonigral neurons of rats with METH-induced DA loss (Daberkow et al., 2008; Barker-Haliski et al., 2012b), and the magnitude of that disruption correlates with the degree of DA loss (Barker-Haliski et al., 2012b), as does the disruption of phasic DA signaling (Bergstrom and Garris, 2003). Our present results thus suggest an intriguing role for DA in general, and perhaps phasic DA signaling through D1 DA receptors in particular, in mediating normal activity-dependent mRNA cytoplasmic localization in striatonigral efferent neurons.

As noted above, phasic DA signaling is disrupted in rats with METH-induced DA loss, and phasic DA signaling is predicted to selectively affect signaling through the D1 subtype of DA receptors. The D1 DA receptor is positively coupled to adenylyl cyclase (Herve et al., 1995; Gerfen and Surmeier, 2011) and, thus, PKA. PKA mediates nuclear export of several cellular factors critically involved in cytoskeletal rearrangement and signaling underlying neuroplasticity processes. For example, PKA induces nuclear export of the MAPK-activated protein kinase, MK5 (Gerits et al., 2007; Kostenko et al., 2011), and such MK5 export induces F-actin rearrangement in PC12 cells (Gerits et al., 2007). Additionally, PKA regulates the nuclear export of the polypyrimidine tract-binding protein (PTB) and association of PTB with β-actin, which is essential to neurite outgrowth in PC12 cells (Ma et al., 2007). Furthermore, PKA phosphorylates histone deacetylase 5 (HDAC5), thereby preventing its nuclear export (Ha et al., 2010), and HDAC5 was recently reported to undergo activity-dependent nucleocytoplasmic shuttling within cultured hippocampal neurons (Schlumm et al., 2013). Thus, the regulation of PKA by DA D1 receptor activation is an attractive target for exploration of the processes potentially mediating the differential subcellular distribution of IEG mRNAs in striatal efferent neuron subpopulations.

In model organisms, distribution of mRNA-protein (mRNP) complexes from the nucleus to the cytoplasm requires complex intracellular signaling and could thus be a critical processing point whereby neuronal mRNA transport is regulated (Oeffinger and Zenklusen, 2012). Most evidence suggests that export through the nuclear pore complex (NPC) itself occurs relatively quickly in metazoans (Ribbeck and Gorlich, 2001; Smith et al., 2002). However, once in the cytoplasm, exported mRNPs are prevented from returning to the nucleus by the DEAD-box RNA helicase DDX19 (Tran et al., 2007; Stewart, 2010; Folkmann et al., 2011). DDX19 requires local activation by GLE1 and its co-factor inositol hexakisphosphate (IP6), to stimulate RNA binding necessary for cytoplasmic mRNP remodeling and retention (Tran et al., 2007; Hodge et al., 2011; Montpetit et al., 2011). IP6 is highly expressed in striatum, can be activated in response to electroconvulsive shock, and can activate PKA (Yang et al., 2001). Thus, while export through the NPC is relatively fast, cytoplasmic retention of mRNPs such as *Arc* and *zif268* may be differentially modulated by IP6-mediated regulation of DDX19 activity within striatal efferent neurons. How such cytoplasmic mRNP remodeling may proceed in neurons, as well as how such cytoplasmic directionality could contribute to *Arc* (Daberkow et al., 2007; Barker-Haliski et al., 2012b) and *zif268* phenotypic expression differences within striatal efferent neurons, is currently unknown, but could be a basis for the phenotypic differences in cytoplasmic mRNA expression.

Our prior observations that more striatonigral than striatopallidal neurons contain cytoplasmic *Arc* mRNA suggested divergent mRNA regulatory mechanisms within these efferent neuron subpopulations (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b). Furthermore, our present observations emphasize that this cytoplasmic expression difference is not unique to *Arc*, but may rather be a function of global differences in IEG

mRNA regulation within striatal efferent neuron subpopulations. Additionally, we demonstrate that partial DA depletion significantly disrupts cytoplasmic localization dynamics, thus suggesting a role for DA in mediating normal posttranscriptional mRNA regulation in striatal neurons. While much of our understanding of mRNA regulation and synaptic plasticity processes in general have been skillfully elucidated in hippocampal preparations (Guzowski et al., 1999; Guzowski et al., 2006; Bramham et al., 2008; Lebeau et al., 2008; Bramham et al., 2009), it is essential that such meticulous studies are similarly performed in striatal efferent neurons given the recent findings demonstrating vast differences in synaptic plasticity-associated factor expression between striatum and hippocampus (Baucum et al., 2013). Our present results suggest that while much work has already demonstrated significant signaling differences between striatal efferent neurons (Surmeier et al., 2009; Gerfen and Surmeier, 2011; Kreitzer and Berke, 2011), additional investigation into the posttranscriptional regulation of mRNA expression in striatal efferent neuron subpopulations will be necessary to fully understand how these neuronal phenotypes modulate synaptic plasticity signaling processes under both normal, as well as aberrant, conditions. Such investigations into the subcellular mechanisms underlying neuroplasticity in striatal efferent neurons will be critical for advancing our ability to prevent or manage neurological and neuropsychiatric consequences of basal ganglia dysfunction.

Acknowledgements

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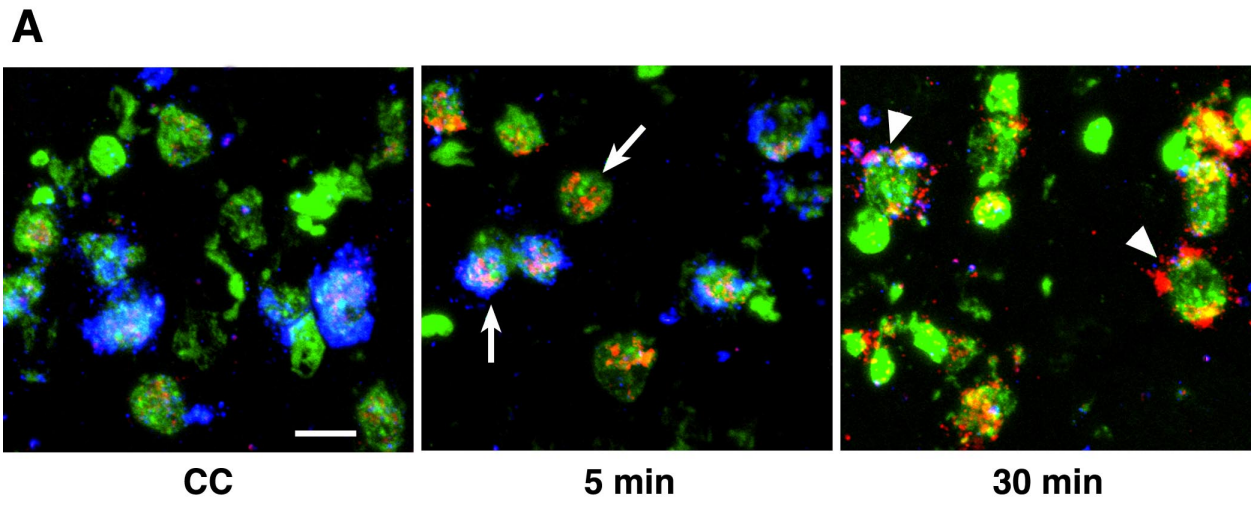
Figure 3.1. *Activity-dependent expression of zif268 mRNA within striatal efferent neurons of normal rats.* A) Representative photomicrographs from *in situ* hybridization histochemistry for *zif268* and *ppe* mRNA in dorsomedial striatum in caged control animals (CC), or after spatial exploration of a novel environment for 5 minutes then immediate sacrifice ("5-min"), or sacrifice 25 minutes later ("30-min"). Arrows indicate neurons positive for *zif268* mRNA intranuclear foci; arrowheads indicate neurons positive for *zif268* mRNA cytoplasmic localization. Blue is *preproenkephalin* mRNA, red is *zif268* mRNA, and green is SYTOX nuclear counterstain. Scale bar = 10 μm B) Numbers of cells with intranuclear *zif268* foci following spatial exploration of a novel environment. Data are mean (± SEM; 8-9 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *zif268* and *preproenkephalin* mRNAs. C) Numbers of cells with cytoplasmic *zif268* expression following spatial exploration of a novel environment. Data are mean (± SEM; 8-9 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *zif268* and *preproenkephalin* mRNAs. * Significant main effect of time. # Significant effect of phenotype at 30-min.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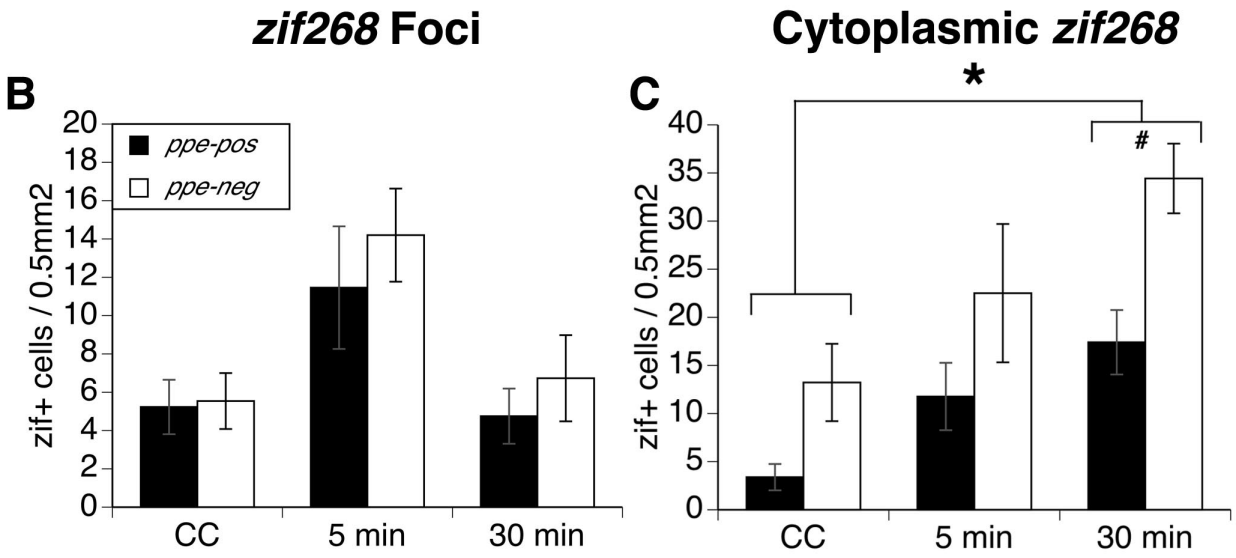
zif268 Foci
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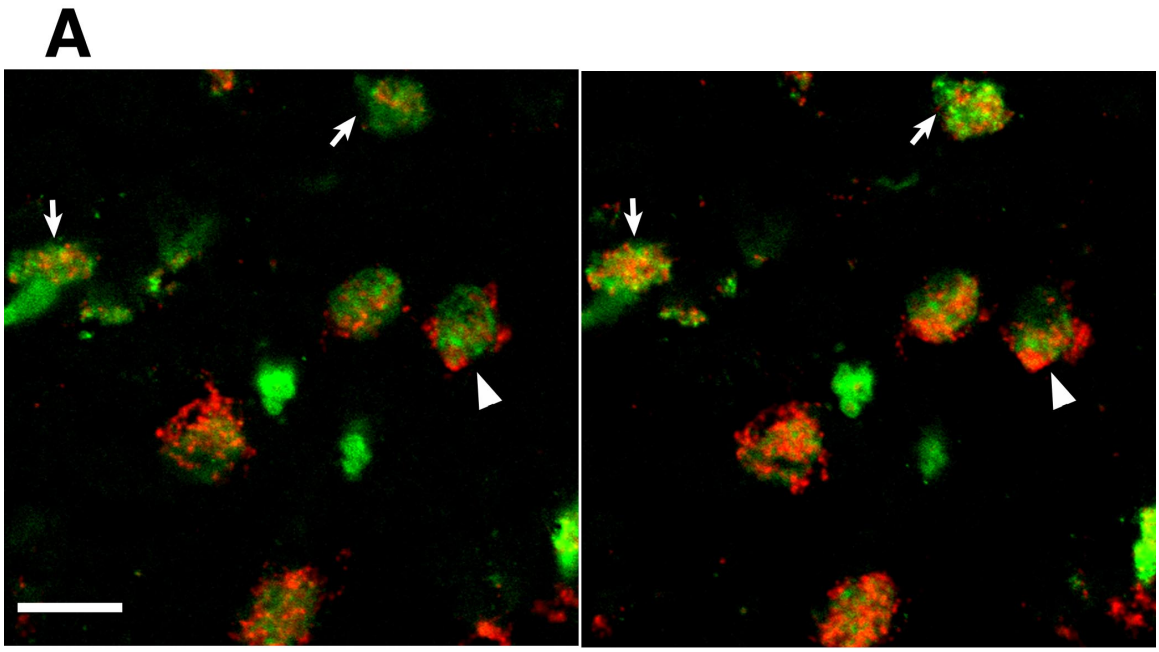
Figure 3.2. *Striatal efferent neurons show differences in numbers of cells with cytoplasmic localization of zif268 mRNA 30 minutes after spatial exploration of a novel environment.* A) Representative photomicrographs of two consecutive confocal *z*-stacks illustrating "full" nucleus labeling (arrows), "whole cell" labeling (arrowheads), or "cytoplasmic" labeling (asterisks). Red is *zif268* mRNA and green is SYTOX nuclear counterstain. Scale bar = 10 μm. B) Striatopallidal, *ppe*-pos neurons are more frequently positive for nuclei "full" of *zif268* mRNA 30 minutes after spatial exploration of a novel environment, whereas striatonigral, *ppe*-neg neurons are more frequently positive for "whole cell" or "cytoplasmic"-only *zif268* mRNA labeling. Data are mean (± SEM; 8-9 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *zif268* and *preproenkephalin* mRNAs. # Significantly greater than *ppe*-neg neurons. * Significantly greater than *ppe*-pos neurons.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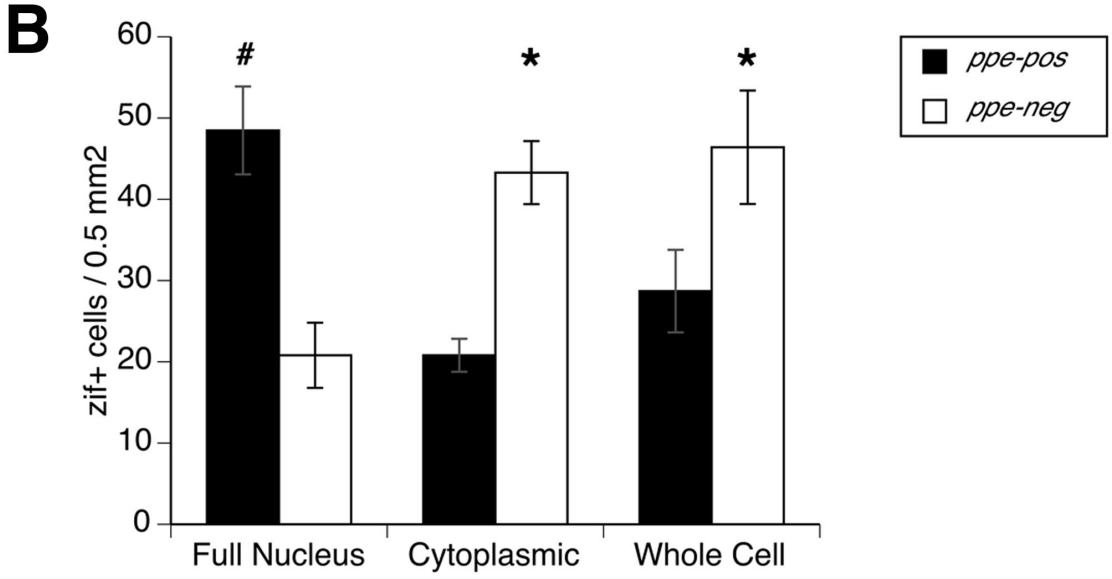
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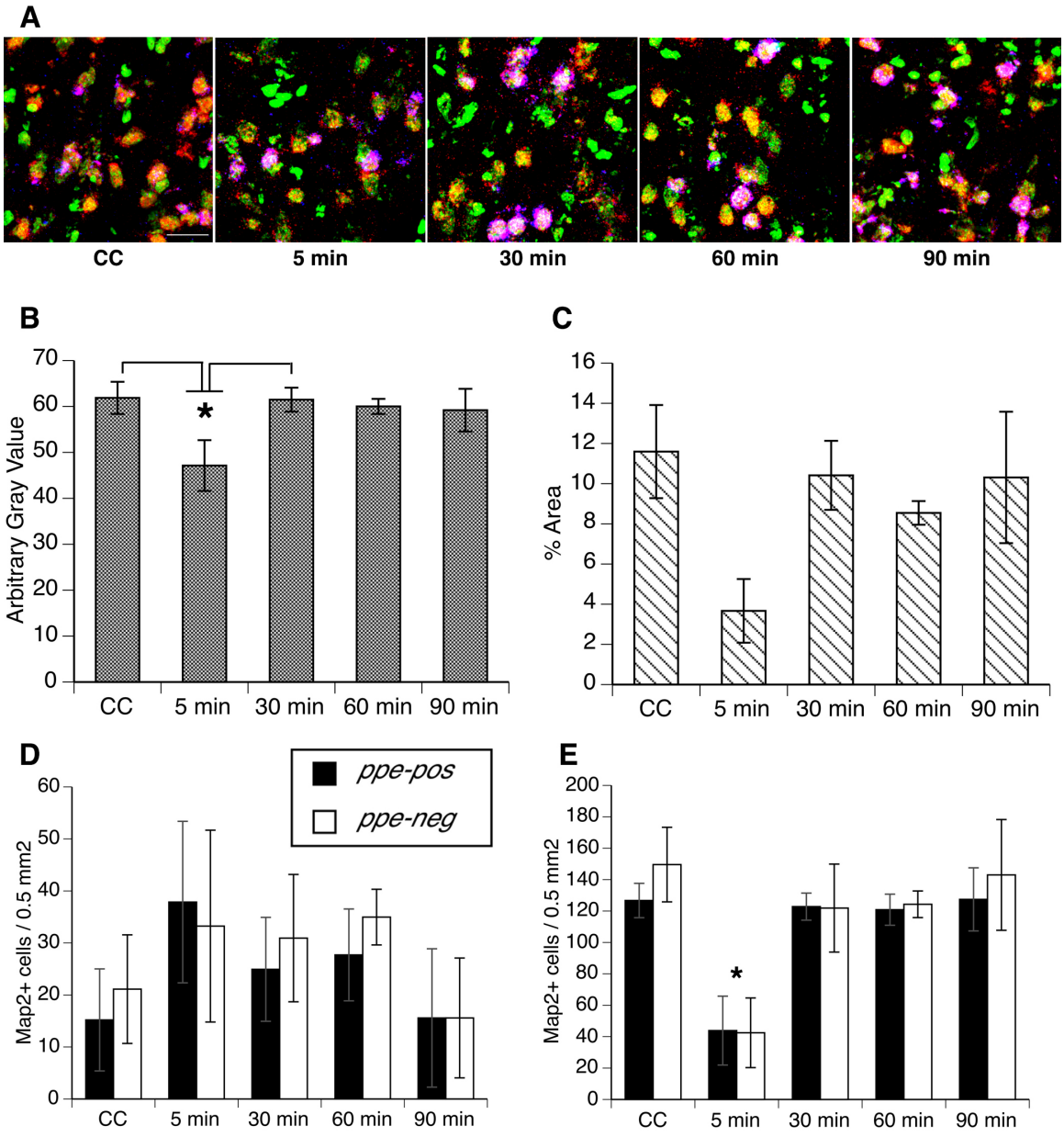
ppe-pos
ppe-neg

Figure 3.3. *There is no phenotypic difference in the numbers of cells with cytoplasmic localization of constitutively expressed Map2 mRNA.* A) Representative photomicrographs of *Map2* and *ppe* mRNA within efferent neurons of dorsomedial striatum following spatial exploration of a novel environment. Rats were either immediately sacrificed as caged controls (CC), or allowed to explore a novel environment for 5 minutes then immediately sacrificed ("5-min"), or sacrificed 25 minutes later ("30-min"), 55 minutes later ("60-min") or 85 minutes later ("90-min"). Blue is *preproenkephalin* mRNA, red is *Map2* mRNA, and green is SYTOX nuclear counterstain. B) Densitometric analysis of average *Map2* pixel intensity in dorsomedial striatum. *Significantly different from CC and 30-min. C) Percent image area with *Map2*-positive pixels in dorsomedial striatum. D) Numbers of labeled neurons with cytoplasmic *Map2* mRNA following spatial exploration of a novel environment. Data are mean (± SEM; 4 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *Map2* and *preproenkephalin* mRNAs. E) Numbers of labeled neurons with whole cell *Map2* mRNA labeling following spatial exploration of a novel environment. Data are mean (± SEM; 4 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *Map2* and *preproenkephalin* mRNAs. * Significantly different from numbers of neurons in CC and 30-min groups. Scale bar = 25 μm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Map2+ cells / 0.5 mm2
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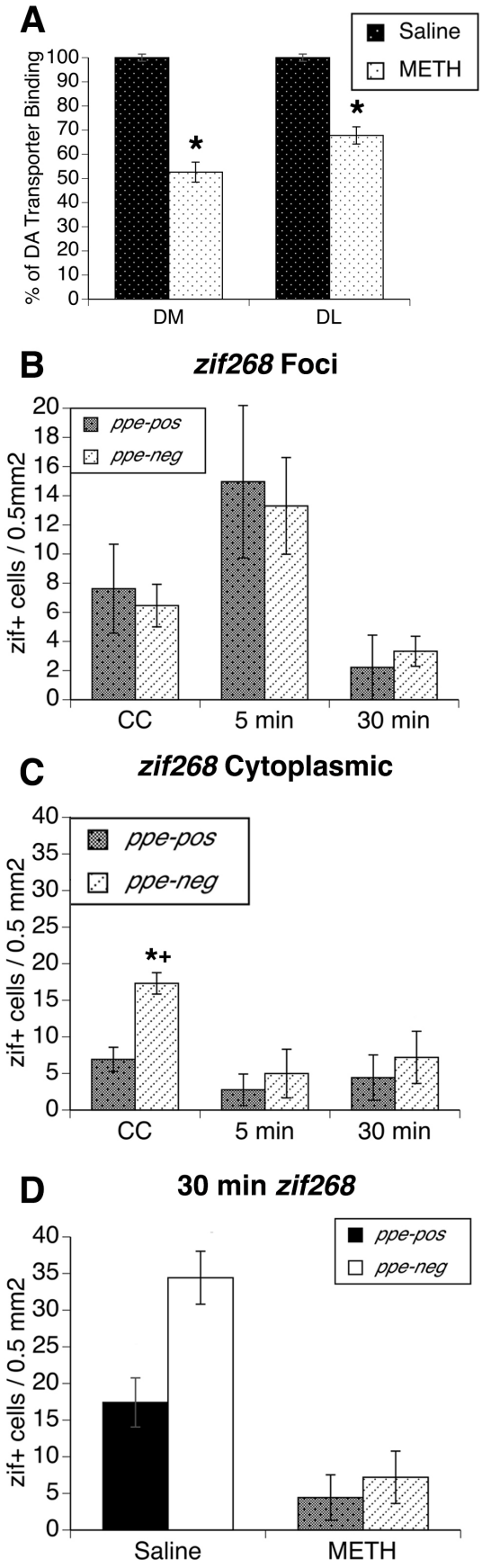
Figure 3.4. *Activity-dependent zif268 mRNA expression within striatal efferent neurons of rats with partial monoamine depletion induced by METH.* A) Three weeks after a neurotoxic regimen of METH, METH-pretreated rats have significant decreases in dorsomedial (DM) and dorsolateral (DL) striatum DA transporter binding relative to saline-pretreated controls. * Significantly less than saline-pretreated rats. B) Numbers of labeled neurons with intranuclear *zif268* foci following spatial exploration of a novel environment in METH-pretreated rats. Data are mean (± SEM; 5-12 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *zif268* and *preproenkephalin* mRNAs. C) Numbers of labeled neurons with cytoplasmic *zif268* expression following spatial exploration of a novel environment in METH-pretreated rats. Data are mean (± SEM; 5-12 animals / time point) numbers of neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization for *zif268* and *preproenkephalin* mRNAs. * Significantly different from numbers of *ppe*-neg neurons in 5- and 30-min groups. + Significantly different from numbers of *ppe*-pos neurons in CC group. D) Comparing saline- and METH-pretreated rats 30 minutes after novel environment exploration reveals profound disruption of cytoplasmic mRNA distribution subsequent to METH-induced partial monoamine depletion.


A
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% of DA Transporter Binding
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zif268 Foci
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Saline
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CHAPTER 4

DISRUPTION OF SUBCELLULAR ARC/ARG3.1 MRNA EXPRESSION IN STRIATAL EFFERENT NEURONS FOLLOWING PARTIAL MONOAMINE LOSS INDUCED BY METHAMPHETAMINE

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ORIGINAL ARTICLE

Disruption of subcellular *Arc/Arg 3.1* mRNA expression in striatal efferent neurons following partial monoamine loss induced by methamphetamine

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Abstract

The immediate-early gene *Arc* (activity-regulated cytoskeleton-associated protein) is provocative in the context of neuroplasticity because of its experience-dependent regulation and mRNA transport to and translation at activated synapses. Normal rats have more *preproenkephalin*-negative (*ppe*-neg; presumed striatonigral) neurons with cytoplasmic *Arc* mRNA than *ppe*-positive (*ppe*-pos; striatopallidal) neurons, despite equivalent numbers of these neurons showing novelty-induced transcriptional activation of *Arc*. Furthermore, rats with partial monoamine loss induced by methamphetamine (METH) show impaired *Arc* mRNA expression in both *ppe*-neg and *ppe*-pos neurons relative to normal animals following response-reversal learning. In this study, *Arc* expression induced by exposure to a novel environment was used to assess transcriptional activation and cytoplasmic localization of *Arc* mRNA in striatal efferent neuron subpopulations subsequent to METH-induced neurotoxicity. Partial monoamine depletion significantly altered *Arc* expression. Specifically, basal *Arc* expression was elevated, but novelty-induced transcriptional activation was abolished. Without novelty-induced *Arc* transcription, METH-pre-treated rats also had fewer neurons with cytoplasmic *Arc* mRNA expression, with the effect being greater for *ppe*-neg neurons. Thus, METH-induced neurotoxicity substantially alters striatal efferent neuron function at the level of *Arc* transcription, suggesting a long-term shift in basal ganglia neuroplasticity processes subsequent to METH-induced neurotoxicity. Such changes potentially underlie striatally based learning deficits associated with METH-induced neurotoxicity.

Keywords: Arc/Arg 3.1, dopamine, immediate-early gene, methamphetamine, striatum.
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Methamphetamine (METH) is a highly addictive psychostimulant that can induce substantial forebrain dopamine (DA) and serotonin depletions when administered at high doses (Wagner *et al.* 1980; Woolverton *et al.* 1989). Although METH-induced monoamine depletions are significantly less than those observed in Parkinson's disease, these partial depletions lead to impairments in basal ganglia-mediated behavioral and cognitive abilities in both rodents and humans (Chapman *et al.* 2001; Volkow *et al.* 2001; Kalechstein *et al.* 2003; Johanson *et al.* 2006). Furthermore, recent data show that individuals with a history of methamphetamine or other amphetamine abuse are more likely to develop Parkinsonism (Callaghan *et al.* 2012), suggesting that METH exposure may be associated with a significant pre-clinical period of partial striatal DA loss and associated consequences.

The bases for the cognitive and behavioral sequelae of METH-induced partial DA loss remain unknown. Approximately, 95% of neurons in striatum are medium spiny neurons, and these efferent neurons can be divided into

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Abbreviations used: Arc/Arg3.1, activity-regulated cytoskeleton-associated protein; CC, caged control rats; DA, dopamine; DAT, dopamine transporter; DIG-UTP, digoxigenin-UTP; DL, dorsolateral; DM, dorsomedial; ERK1/2, extracellular signal-regulated kinase; FITC-UTP, fluorescein-UTP; LTD, long-term depression; LTP, long-term potentiation; METH, (±)-methamphetamine hydrochloride; *ppe*, *preproenkephalin*.

two equal subpopulations: striatonigral 'direct' pathway neurons and striatopallidal 'indirect' pathway neurons (Wichmann and DeLong 1996; Obeso *et al.* 1997; Grillner *et al.* 2005). These efferent neurons can be phenotypically differentiated at the cell body level by their selective expression of neuropeptides: striatopallidal neurons express *preproenkephalin* (*ppe*-pos), whereas striatonigral neurons do not [*ppe*-neg (Gerfen and Young 1988)]. Striatonigral neurons predominately express D1-DA receptors, whereas striatopallidal neurons predominately express D2-DA receptors. Prior work suggests that METH-induced functional impairments are substantially more pronounced in striatonigral neurons (Chapman *et al.* 2001; Johnson-Davis *et al.* 2002; Daberkow *et al.* 2008), consistent with findings reported for rats with partial DA loss induced by 6-hydroxydopamine (Nisenbaum *et al.* 1996). Such changes may be because, in part, of impairments in phasic DA signaling associated with the METH-induced neurotoxicity (Howard *et al.* 2011), as the D1-DA receptor may be particularly sensitive to such changes (Floresco *et al.* 2003; Dreyer *et al.* 2010).

The immediate-early gene *Arc/Arg3.1* (activity-regulated cytoskeleton-associated protein) is a critical mediator of normal neuroplasticity processes and is induced by N-methyl-D-asparate (NMDA)-receptor activation before being trafficked to activated synapses, where it is locally translated (Lyford *et al.* 1995; Bramham *et al.* 2008). *Arc* mRNA expression is induced in an experience-dependent manner (Guzowski *et al.* 1999; Daberkow *et al.* 2007) within GABAergic striatal neurons (Vazdarjanova *et al.* 2006). Arc protein itself is critical to modulating excitatory glutamatergic inputs via endocytosis of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-type glutamate receptors (Chowdhury *et al.* 2006; Rial Verde *et al.* 2006) and to maintaining synaptic plasticity via modulation of long-term potentiation (LTP) and long-term depression (LTD) (Bloomer *et al.* 2008; Bramham *et al.* 2008). Blocking *Arc* translation via antisense oligonucleotide infusions impairs consolidation of learning (Guzowski *et al.* 2000), including basal ganglia-mediated learning (Hearing *et al.* 2011; Pastuzyn *et al.* 2012). Thus, *Arc* is critical for consolidation of learning, including those processes mediated by dorsal striatum.

We have previously reported that despite equivalent numbers of neurons showing transcriptional activation of the *Arc* gene, there are more striatonigral neurons with *Arc* mRNA in the cytoplasm than striatopallidal neurons in normal animals following exploration of a novel environment (Daberkow *et al.* 2007) – the prototypical behavioral paradigm used to assess *Arc* expression (Guzowski *et al.* 1999; Chawla *et al.* 2005; Vazdarjanova *et al.* 2006). Furthermore, we have previously reported that rats with prior exposure to a neurotoxic regimen of METH show decreased numbers of striatal neurons with cytoplasmic *Arc* mRNA expression after engaging in a striatally-mediated, response-reversal learning task, with the greatest impairment being in the numbers of striatonigral neurons (Daberkow *et al.* 2008). Also, the correlation between *Arc* mRNA expression in dorsomedial (DM) striatum and trials to criterion on the response-reversal learning task observed in normal animals (Daberkow *et al.* 2007, 2008) is lost in METH-pre-treated animals (Daberkow *et al.* 2008). Furthermore, reversal learning in METH-pre-treated rats is no longer sensitive to antisense oligonucleotide-mediated disruption of *Arc* translation in DM striatum (Pastuzyn *et al.* 2012). However, the paradigms examined to date do not allow us to discern whether the decreased numbers of striatal neurons with *Arc* mRNA expression in the cytoplasm in METH-pre-treated rats arises because of deficits in transcriptional activation of the *Arc* gene or deficits in the trafficking of *Arc* mRNA into the cytoplasm. Thus, the goal of the present studies was to determine whether METH-induced neurotoxicity is associated with acute disruption of *Arc* transcriptional activation versus longer duration processes regulating *Arc* trafficking/cytoplasmic stability. Furthermore, an additional goal of the present work was to examine further whether there are phenotypic differences in the acute regulation of *Arc* mRNA transcriptional activation and cytoplasmic expression following METH-induced neurotoxicity so as to better define how partial DA depletion impacts upon striatal efferent neuron function. Clarifying the impact of METH-induced neurotoxicity on the transcriptional activation and cytoplasmic regulation of *Arc* mRNA in striatum is critical so as to better inform treatment strategies to ameliorate cognitive and behavioral impairments in human METH abusers (Volkow *et al.* 2001; Kalechstein *et al.* 2003; Johanson *et al.* 2006).

Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC, USA; 275–300 g) were singly housed in tub cages in a room controlled for temperature and lighting (12 : 12 h). All animal care and experimental procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (8th Ed.), followed the ARRIVE guidelines (Kilkenny *et al.* 2010) and were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Methamphetamine treatment

Rats were treated with (±)-methamphetamine hydrochloride (NIDA, Research Triangle Park, NC, USA) as previously described (Daberkow *et al.* 2008; Son *et al.* 2011). This specific regimen was chosen to model METH-induced damage to striatal monoamine systems. Although METH self-administration models may more closely mimic human exposure by self-administration, such contingent models typically do not recapitulate long-lasting striatal DA toxicity (Schwendt *et al.* 2009; Brennan *et al.* 2010; McFadden *et al.* 2012; Reichel *et al.* 2012), perhaps because of the duration of

the exposure, as one study (Krasnova *et al.* 2010), with much longer daily access (15 h/day for 8 days) did report DA neuron toxicity. Thus, the single day, non-contingent binge regimen implemented in this study serves as a more rapid and reproducible model with face validity to the striatal DA depletions present in individuals with a history of METH exposure and who may, in fact, be in a pre-clinical Parkinsonian state. On the METH treatment day, rats were housed five to six animals per cage. Rats received four injections of either (±)-METH (10 mg/kg free base, s.c.) or 0.9% saline (1 mL/kg, s.c.) at 2-h intervals, with rectal temperatures recorded hourly (Fig. 1a). If core temperature exceeded 41°C, the rat was removed and placed in a separate cage over ice to decrease hyperthermia. Twelve hours after the final injection, rats were returned to home cages and given free access to food and water until behavioral manipulations 3 weeks later.

Behavioral activation via novel environment exploration

Rats were divided into three experimental groups ['5-min', '30-min', caged controls (CC)], each consisting of 6–8 saline- or 10–11 METH-pre-treated rats. After initially observing elevated basal *Arc* expression in CC METH rats, an additional seven rats were treated with METH as described above and killed as CC. Thus, the METH CC group consisted of 18 animals. Rats were removed from their home cage after having been isolated for 24 h. CC rats were killed immediately upon removal from the home cage. Rats in the '5-min' group were exposed to a novel environment [50 × 40 × 40 cm tall Rubbermaid tub with pictures on two walls, as previously described (Daberkow *et al.* 2007; Guzowski *et al.* 1999)] for 5 min, then immediately killed. Rats in the '30-min' group were exposed for 5 min to the novel environment, returned to the home cage for 25 min, and then killed. Animals were killed by CO_2 exposure, decapitated, and brains immediately removed and flash-frozen in 2-methylbutane (Mallinckrodt Baker, Phillipsburg, NJ, USA) chilled on dry ice.

Tissue preparation

Twelve-micrometer cryosections of striatum [Cambridge Instruments, Bayreuth, Germany; Bregma: +1.60 to −0.8 mm (Paxinos and Watson, 1998)] were thaw-mounted onto SuperFrost Plus slides (VWR, Batavia, IL, USA), then post-fixed as previously described (Ganguly and Keefe 2001).

DAT Autoradiography

METH-induced DA depletions in dorsal striatum were analyzed using [I^{125}]RTI-55 binding to the dopamine transporter (DAT), as described previously (Boja *et al.* 1992; Pastuzyn *et al.* 2012). DAT binding levels were normalized to percent of saline controls for DM and dorsolateral (DL) striatum (Fig. 1b).

Fluorescent in situ hybridization for Arc/ppe mRNAs

Expression of *Arc* mRNA in striatal efferent neurons was determined by double-label fluorescence *in situ* hybridization histochemistry (FISH) for *Arc* and *ppe* mRNAs, as previously described (Daberkow *et al.* 2007, 2008). Full-length ribonucleotide probes complementary to mRNAs for *Arc* (Lyford *et al.* 1995) and *ppe* (Yoshikawa *et al.* 1984) were synthesized from cDNAs using digoxigenin-UTP (DIG-UTP) and fluorescein-UTP (FITC-UTP) with T7 and SP6 RNA polymerases and DIG- and FITC-UTP RNA-labeling kits, respectively (Roche Applied Science, Indianapolis, IN, USA). Ribonucleotide probes were hybridized and detected as previously described (Daberkow *et al.* 2007, 2008).

Image acquisition

DAT film autoradiograms were digitized, and four sections per animal in rostral (+1.6 mm from bregma), middle (+0.5 mm from bregma), and caudal (−0.8 mm from bregma) dorsal striatum analyzed with *ImageJ* (Pastuzyn *et al.* 2012).

For FISH, a 0.18 mm^2 montage from DM striatum was captured with an FV1000 confocal laser-scanning microscope (Olympus, Center Valley, PA, USA) with motorized stage (Prior Scientific, Rockland, MA, USA) using a 60x, 1.45 NA oil-immersion lens (plan APO) and 488-nm Ar and 543-nm and 633-nm HeNe lasers. Areas of analysis were *z*-sectioned in 1-μm-thick optical sections (10 *z*-sections per image) (Daberkow *et al.* 2007, 2008). The numbers of *ppe*-pos and *ppe*-neg neurons with *Arc* mRNA staining in the nucleus and in the cytoplasm were determined, as previously described (Guzowski *et al.* 1999; Daberkow *et al.* 2007, 2008).

Statistical analysis

Analysis of DAT binding was accomplished by two-sample *t*-tests. METH and saline effects on body temperature were analyzed by MANOVA. Expression of *Arc* mRNA in each subcellular compartment was first compared across saline- and METH-pre-treated rats using MANOVA (treatment × time × phenotype), although for clarity, *Arc* expression data are shown in separate, side-by-side graphs for saline- and METH-pre-treated rats (Figs 3, 4). *Post hoc* analyses of significant interactions were achieved via Tukey–Kramer test for between-subjects factors (treatment, time) or *post hoc* t-tests for the within-subjects factor (phenotype). For all analyses, p-values < 0.05 were significant.

Results

METH-induced partial dopamine depletions

Treatment with METH resulted in hyperthermia necessary to produce long-term neurotoxicity (Ali *et al.* 1994). METH-treated rats reached maximum temperatures of 39.7°C (± 0.8°C; Fig. 1a), whereas saline-treated rats achieved maximum temperatures of 36.8°C (± 0.5°C). There were significant main effects of treatment ($F_{(1,54)} = 192.9$, $p < 0.0001$) and time ($F_{(3,54)} = 102.1$, $p < 0.0001$) and a significant time × treatment interaction ($F_{(3,54)} = 113.0$, $p < 0.0001$). At all time points after baseline, core temperatures in METH-treated rats were significantly elevated over saline (baseline, $p = 0.3$; temp 1-temp 7, $p < 0.0001$). Three weeks later, DAT binding in DM striatum was 57.2 ± 4.2% of control (mean ± SEM; $n = 40$ METH, $n = 23$ saline; $t_{(1,26)} = -9.51$, $p < 0.0001$), and that in DL striatum was 65.5 ± 3.7% of control ($t_{(1,26)} = -10.14$, $p < 0.0001$; Fig. 1b).

METH-induced neurotoxicity is associated with impaired transcriptional activation of Arc mRNA

Using catFISH (Guzowski *et al.* 1999; Daberkow *et al.* 2007, 2008), we determined the subcellular localization of *Arc* mRNA in striatal efferent neurons following spatial

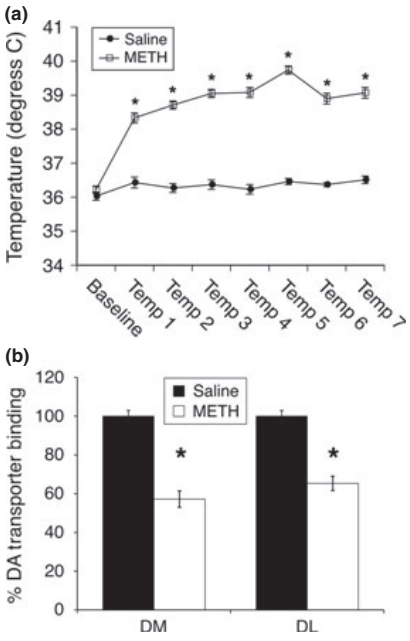

Fig. 1 Methamphetamine pre-treatment and subsequent dopamine (DA) depletions. (a) A neurotoxic regimen of methamphetamine (METH; 4 × 10 mg/kg) leads to a significant elevation of core body temperature relative to saline-treated animals (treatment effect $F_{(1,54)} = 192.9$, $p < 0.0001$). (b) METH-pre-treated rats ($n = 40$) show significant DA depletions in both dorsomedial (DM) and dorsolateral (DL) striatum as measured by [^{125}I]RTI-55 dopamine transporter autoradiography 3 weeks later [mean gray values (arbitrary units) ± SEM expressed as% of saline control ($n = 23$)]. *Significantly different from saline control, $p \leq 0.05$.

exploration of a novel environment (Fig. 2a–f). As previously reported in normal animals (Guzowski *et al.* 1999; Daberkow *et al.* 2007), exposure to the novel environment increased nuclear *Arc* mRNA expression within 5 min (Figs 2 and 3a). MANOVA revealed significant main effects of time ($F_{(2,54)} = 6.0$, $p < 0.005$) and treatment ($F_{(1,54)} = 5.96$, $p < 0.02$) and a significant time x treatment interaction ($F_{(2,54)} = 4.99$, $p < 0.02$). There was no main effect of phenotype ($F_{(1,54)} = 2.72$, $p = 0.1$) and no phenotype × time ($F_{(2,54)} = 0.7$, $p = 0.5$), phenotype × treatment ($F_{(1,54)} = 0.5$, $p = 0.5$) or phenotype × time × treatment ($F_{(2,54)} = 0.6$, $p = 0.6$) interactions. *Post hoc* analysis of the time × treatment interaction revealed that in saline-pre-treated rats the number of cells with nuclear *Arc* mRNA expression was significantly greater in the 5-min group than in the CC ($p < 0.01$) or 30-min ($p < 0.001$) groups. There were no such effects in METH-pre-treated rats, as the numbers of cells with intranuclear foci of *Arc* mRNA in the METH-pre-treated groups were not different from each other ($p > 0.1$). However, overall (main effect of treatment) there were more cells with nuclear *Arc* mRNA in the METH- versus saline-pre-treated rats ($p < 0.02$; Fig. 3a vs. b). Thus, rats with METH-induced neurotoxicity have higher basal *Arc* transcription, but fail to induce *Arc* transcription in response to behavioral activation.

METH-induced neurotoxicity is associated with impaired cytoplasmic Arc mRNA expression

As previously reported (Daberkow *et al.* 2007), saline-pre-treated rats in the 30-min group had a significant increase in the number of striatal efferent neurons with *Arc* mRNA in the cytoplasm (Figs 4, 5). Furthermore, as we have previously reported (Daberkow *et al.* 2007, 2008), normal animals had more striatonigral (*ppe*-neg) than striatopallidal (*ppe*-pos) neurons with cytoplasmic *Arc* mRNA expression (Figs 4 and 5a). Specifically, MANOVA revealed a significant main effect for time ($F_{(2,54)} = 16.9$, $p < 0.0001$) and significant time × treatment ($F_{(2,54)} = 3.3$, $p < 0.05$) and phenotype × time × treatment ($F_{(2,54)} = 3.8$, $p < 0.03$) interactions. There was no significant main effect of treatment ($F_{(1,54)} = 0.7$, $p = 0.4$) or phenotype ($F_{(1,54)} = 3.1$, $p = 0.08$) and no significant phenotype × time ($F_{(2,54)} = 0.6$, $p = 0.5$) or phenotype × treatment ($F_{(1,54)} = 0.8$, $p = 0.4$) interactions. *Post hoc* analysis of the time × treatment interaction revealed greater numbers of neurons with cytoplasmic *Arc* mRNA expression in saline-pre-treated, 30-min rats relative to saline-pre-treated, CC ($p = 0.002$) and 5-min ($p < 0.001$) groups, consistent with previous reports regarding the time course of normal *Arc* trafficking (Guzowski *et al.* 1999; Daberkow *et al.* 2007). Conversely, there were no significant differences between the numbers of cells with cytoplasmic *Arc* mRNA expression in the METH-pre-treated, 30-min group relative to the METH-pre-treated, CC ($p = 0.6$) and 5-min ($p = 0.1$) groups, again indicating a general overall lack of *Arc* mRNA induction associated with spatial exploration in METH-pre-treated rats.

To examine the phenotypic differences, *post hoc* t-tests were performed on the numbers of *ppe*-neg and *ppe*-pos neurons with cytoplasmic *Arc* mRNA in saline- and METH-pre-treated rats. Paired t-tests confirmed (Daberkow *et al.* 2007) greater numbers of both *ppe*-neg ($t = 3.9$, $p = 0.003$) and *ppe*-pos ($t = 3.9$, $p = 0.001$) neurons with cytoplasmic *Arc* in the saline-pre-treated, 30-min group relative to saline-pre-treated, CC group (Fig. 4a) and that there were more *ppe*-neg than *ppe*-pos neurons with cytoplasmic *Arc* mRNA expression in the saline-pre-treated, 30-min group (Figs 4a and 5a; $t = 2.5$, $p = 0.02$). In METH-pre-treated, CC rats, there was no significant difference from saline-pre-treated, CC rats in the numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA (Fig. 4; $t = -0.28$, $p > 0.1$). Additionally, in METH-pre-treated rats, there was no significant increase in numbers of *ppe*-neg neurons with cytoplasmic *Arc* at 30 min (Fig. 4b; $t = 0.44$, $p = 0.31$) relative to METH-pre-treated, CC group, whereas there was a significant increase in the numbers of *ppe*-pos neurons (Fig. 4b; $t = 2.2$, $p = 0.02$).

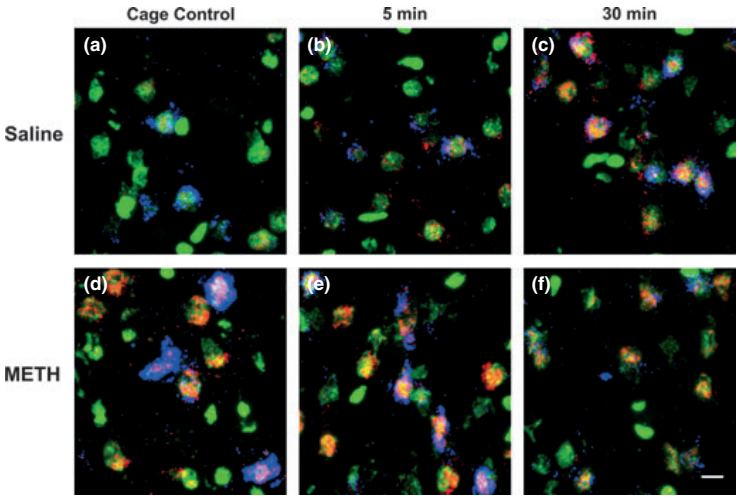

Fig. 2 Representative photomicrographs of *Arc* mRNA expression in rat dorsomedial striatum. Representative *in situ* hybridization histochemical images from rats pre-treated with saline [4 × 1 mL/kg; (a–c) or a neurotoxic regimen of (±)-methamphetamine (METH; 4 × 10 mg/kg, s.c. at 2-h intervals] and then either killed immediately upon removal from the home cage ('caged control'; a, d), allowed to explore a novel environment for 5 min and then killed (5 min; b, e), or allowed to explore the novel environment for 5 min before being returned to the home cage for 25 min before being killed (30 min; c, f). Green is Sytox nuclear counter-stain, blue is *preproenkephalin* mRNA and red is *Arc* (activity-regulated cytoskeleton-associated) mRNA expression. Scale bar = 10 μm.

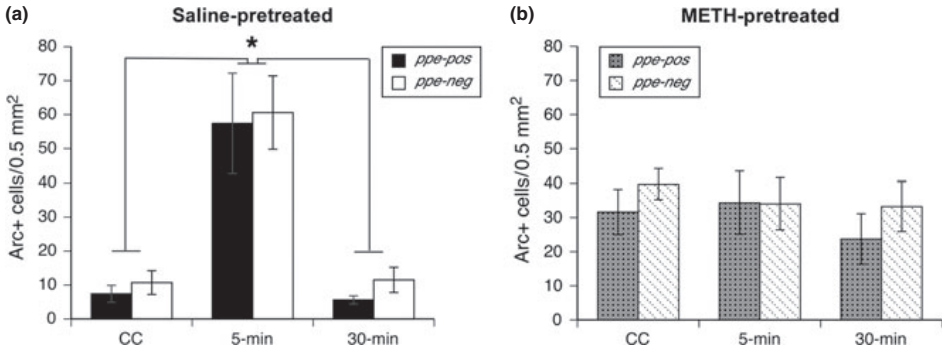

Fig. 3 Nuclear *Arc* mRNA expression in striatal efferent neurons. Data are mean (± SEM; saline $n = 6$–8; METH $n = 10$–18 per time point) numbers of labeled neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization staining for *preproenkeaphalin* and *Arc* (activity-regulated cytoskeleton-associated) mRNAs in the dorsomedial striata of rats treated approximately 3 weeks prior to killing with (a) saline (4 × 1 mL/kg) or (b) a neurotoxic regimen of (±)-methamphetamine (METH; 4 × 10 mg/kg, s.c. at 2-h intervals). Rats were either killed immediately upon removal from the home cage ('caged control'; CC), allowed to explore a novel environment for 5 min and then killed (5 min), or allowed to explore the novel environment for 5 min before being returned to the home cage for 25 min before being killed (30 min). There is a significant main effect of METH-pre-treatment relative to saline-pre-treatment ($p < 0.02$). *Significant main effect of time (5-min point significantly different from both the CC and 30-min groups).

Thus, in the METH-pre-treated, 30-min group, there was no longer a phenotypic difference between the numbers of *ppe*-neg and *ppe*-pos neurons with cytoplasmic *Arc* (Figs 4b and 5a; $t = -0.95$, $p = 0.82$), but there were significantly fewer *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression relative to the saline-pre-treated, 30-min group (Figs 4b and 5a; $t = 3.0$, $p = 0.004$). Interestingly, there were more *ppe*-pos neurons with cytoplasmic *Arc* mRNA expression in the METH-pre-treated, CC group than in the saline-pre-treated, CC group (Fig. 4; $t = -2.4$, $p = 0.01$), and also slightly more *ppe*-pos neurons with cytoplasmic *Arc* mRNA in METH-pre-treated, 30 min relative to METH-pre-treated, CC rats (Fig. 4b; $t = 1.9$, $p = 0.04$). However, there was no difference between the saline- and METH-pre-treated 30-min groups in the numbers of *ppe*-pos neurons with cytoplasmic *Arc* mRNA expression (Figs 4 and 5a; $t = 0.18$, $p = 0.43$).

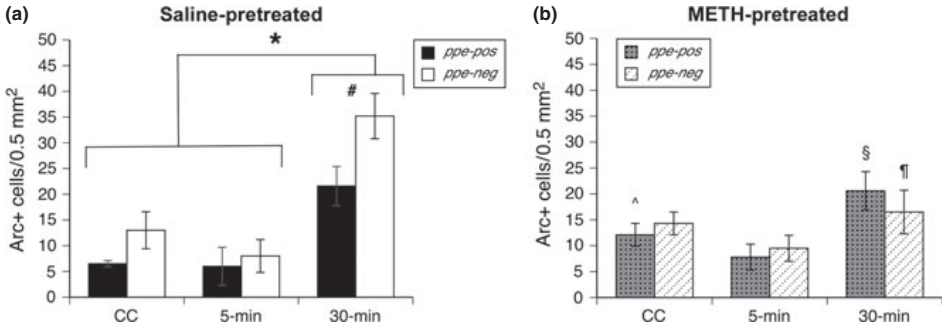

Fig. 4 Cytoplasmic *Arc* mRNA expression in striatal efferent neurons. Data are mean (± SEM; saline $n = 6$–8; METH $n = 10$–18 per time point) numbers of labeled neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization staining for *preproenkeaphalin* and *Arc* (activity-regulated cytoskeleton-associated) mRNAs in the dorsomedial striata of rats treated approximately three weeks prior to killing with (a) saline (4 × 1 mL/kg) or (b) a neurotoxic regimen of (±)-methamphetamine (METH; 4 × 10 mg/kg, s.c. at 2-h intervals). Rats were either killed immediately upon removal from the home cage ('caged control'; CC), allowed to explore a novel environment for 5 min and then killed (5 min), or allowed to explore the novel environment for 5 min before being returned to the home cage for 25 min before being killed (30 min). *Significantly different from all other saline-pre-treated groups, $p \leq 0.05$. #Significantly greater than number of *ppe*-pos neurons in same group. ^Significantly greater than the number of *ppe*-pos neurons with *Arc* mRNA in the cytoplasm in the saline-pre-treated, CC group. §Significantly greater than the number of *ppe*-pos with *Arc* mRNA expression in the cytoplasm in the METH-pre-treated, CC group. ¶Significantly less than the number of *ppe*-neg neurons with *Arc* mRNA in the cytoplasm in the saline-pre-treated, 30-min group.

Upon observing impaired cytoplasmic *Arc* expression in *ppe*-neg neurons in the METH-pre-treated, 30-min group, we assessed whether this impairment was uniformly distributed across METH-pre-treated rats or whether this difference correlated to the DA depletion in each animal. Using only cell counts from the METH-pre-treated, 30-min group, we correlated the numbers of striatal efferent neurons with *Arc* mRNA in the cytoplasm with the percent DA depletion as assessed by DAT autoradiography (Fig. 5b and c). The numbers of *ppe*-pos neurons with cytoplasmic *Arc* mRNA were not significantly correlated with DA loss (Fig. 5b; $R^2 = 0.015$, $p = 0.7$). Conversely, the numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression was significantly inversely correlated to the DA depletion (Fig. 5c; $R^2 = 0.518$, $p = 0.019$), indicating that greater DA loss is associated with fewer *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression. In the METH-pre-treated, 5-min group, there was no significant correlation between cells with nuclear *Arc* and DA depletion (*ppe*-pos $R^2 = 0.005$, $p > 0.5$; *ppe*-neg $R^2 = 0.05$, $p > 0.05$).

Discussion

Herein, we report novel findings that basal *Arc* transcription in striatal efferent neurons is enhanced, but *Arc* mRNA transcriptional activation and cytoplasmic localization in response to behavioral activation is severely impaired, in rats with METH-induced neurotoxicity. Specifically, behavioral activation associated with spatial exploration of a novel environment – the classic paradigm used to examine *Arc* mRNA induction and trafficking (Guzowski *et al.* 1999; Chawla *et al.* 2005; Vazdarjanova *et al.* 2006; Daberkow *et al.* 2007) – resulted in no concomitant increase in cells with nuclear *Arc* expression above the elevated CC levels in METH-pre-treated groups. There was also no significant overall increase in total number of neurons with cytoplasmic *Arc* mRNA in the METH-pre-treated, 30-min group, particularly with respect to *ppe*-neg neurons, similar to the suppressed cytoplasmic expression previously reported following a striatally mediated response-reversal task (Daberkow *et al.* 2008). Interestingly, the degree of DA depletion was significantly negatively correlated with the numbers of *ppe*-neg cells with cytoplasmic *Arc*, suggesting that the loss of this expression may arise as a consequence of disrupted DA signaling in striatum. Taken together, these data reveal a profound dysregulation of *Arc* transcriptional activation and subcellular trafficking. As *Arc* mRNA, and the resulting Arc protein, is critical to normal neuroplasticity processes (Chowdhury *et al.* 2006), such disruption may contribute to disrupted *Arc*-mediated memory processes of the basal ganglia subsequent to METH pre-treatment (Daberkow *et al.* 2008; Pastuzyn *et al.* 2012).

We presently demonstrate an intriguing increase in basal (CC) *Arc* mRNA transcription following METH-induced neurotoxicity relative to saline-pre-treated controls. Persistent changes in basal gene expression are not uncommon after repeated exposure to psychostimulants (c.f. (Unal *et al.* 2009), including reports from our lab showing long-term decreases in *preprotachykinin* mRNA expression after METH-induced neurotoxicity (Chapman *et al.* 2001; Johnson-Davis *et al.* 2002). However, to our knowledge, there are no prior reports of long-term changes in basal immediate-early

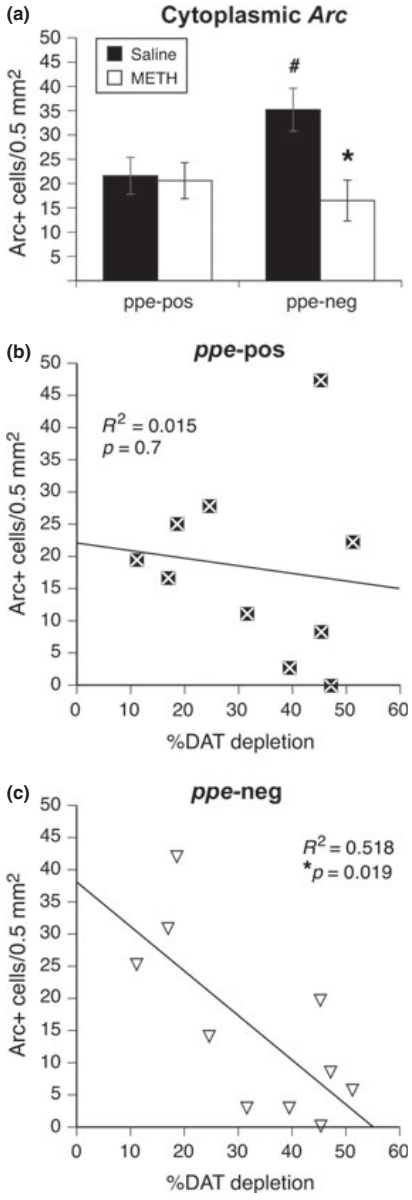

Fig. 5 Cytoplasmic *Arc* mRNA expression in striatal efferent neurons of rats killed 30 min after exposure to a novel environment and relation to degree of methamphetamine-induced dopamine depletion. Partial monoamine depletion significantly only affects the numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA expression. (a) Mean (± SEM; saline $n = 8$; METH $n = 11$) numbers of labeled neurons per 0.5 mm^2 determined from double fluorescent *in situ* hybridization staining for *preproenkeaphalin* and *Arc* (activity-regulated cytoskeleton-associated) mRNAs in the dorsomedial striata of rats treated approximately 3 weeks prior to killing with saline (4 × 1 mL/kg) or a neurotoxic regimen of (±)-methamphetamine (METH; 4 × 10 mg/kg, s.c. at 2-h intervals). These data are the same '30-min' data presented in Fig. 4, but are re-graphed to highlight the differences between the striatal efferent neuron subtypes at this time point. *Significantly different from number of *ppe*-neg neurons in saline-pre-treated rats. #Significantly different from number of *ppe*-pos neurons in saline-pre-treated rats. (b) Correlation between the numbers of *ppe*-pos neurons with *Arc* mRNA in the cytoplasm and the percent DA loss, as assessed by [^{125}I]RTI-55 autoradiography for dopamine transporter binding, induced by the neurotoxic regimen of METH (4 × 10 mg/kg, s.c. at 2-h intervals) three weeks prior to killing. (c) Correlation between the numbers of *ppe*-neg neurons with *Arc* mRNA in the cytoplasm and the percent dopamine (DA) loss, as assessed by [^{125}I] RTI-55 autoradiography for dopamine transporter binding, induced by the neurotoxic regimen of METH (4 × 10 mg/kg, s.c. at 2-h intervals) 3 weeks prior to killing. *Significant correlation, $p = 0.019$.

gene expression in striatal neurons as a consequence of METH-induced neurotoxicity. Given that prior work suggested a relatively preferential impact of METH-induced neurotoxicity on striatonigral neuron function (Chapman *et al.* 2001; Johnson-Davis *et al.* 2002; Daberkow *et al.* 2008), the equivalent increases in basal *Arc* transcription observed in *ppe*-pos and *ppe*-neg neurons were surprising and suggest global alterations in striatal efferent neuron function following METH-induced neurotoxicity. This up-regulation may reflect alterations in signals regulating *Arc* transcription, including brain-derived neurotrophic factor or glutamate. Although both of these molecules are elevated 24–72 h after exposure of animals to a neurotoxic regimen of METH (Thomas *et al.* 2004; Mark *et al.* 2007), whether they remain elevated in striatum weeks later remains unknown. Clearly, further studies are needed to examine the mechanisms underlying long-term dysregulation of basal *Arc* transcription and also the extent to which the expression of other plasticity-related genes is similarly altered.

The present studies also reveal a profound impairment of acute, novelty-induced transcription of *Arc* in rats with METH-induced neurotoxicity. The basis for this observation remains presently unknown, but changes in phasic DA release seem a likely contributing factor. Partial monoamine loss is associated with impaired phasic-like, but not tonic-like, DA release (Bergstrom and Garris 2003; Howard *et al.* 2011). Modeling data suggest that D1-DA receptors should be particularly sensitive to changes in phasic DA amplitude (Dreyer *et al.* 2010). Consistent with this model, phasic-like stimulation of DA transmission increases gene expression in and electrophysiological activity of striatonigral, but not striatopallidal, neurons (Chergui *et al.* 1997; Gonon 1997; Onn *et al.* 2000). Finally, we presently report that in METH-pre-treated rats, the impairment in numbers of *ppe*-neg neurons with cytoplasmic *Arc* mRNA correlates with the extent of DA loss, which is further known to correlate with the degree of impairment of phasic-like DA neurotransmission (Bergstrom and Garris 2003; Howard *et al.* 2011). Thus, impaired phasic DA neurotransmission may ultimately

contribute to the observed deficits in *Arc* expression subsequent to METH-induced neurotoxicity.

Decreased phasic DA neurotransmission and consequent decreases in D1-DA receptor activation may also impair *Arc* transcription by altering upstream signaling cascades. Activation of D1-DA receptors enhances NMDA receptor-mediated currents in striatonigral neurons (Cepeda *et al.* 1993; Andre *et al.* 2010; Jocoy *et al.* 2011) and increases activation of extracellular signal-regulated kinase (ERK1/2) (Valjent *et al.* 2005; Pascoli *et al.* 2011). *Arc* transcription is regulated by NMDA receptor and ERK1/2 activation (Korb and Finkbeiner 2011). Thus, decreased D1-DA receptor activation because of attenuated phasic DA transmission likely results in decreased NMDA receptor and ERK1/2 activation and, thus, decreased activity-dependent *Arc* induction in *ppe*-neg neurons.

Although the above-delineated effects can explain the basis for the impairment of acute, novelty-induced *Arc* transcription in *ppe*-neg neurons, the basis for the impaired transcriptional activation in *ppe*-pos neurons is less apparent. One possibility is that it also arises as a consequence of impaired phasic DA signaling and decreased D1-DA receptor activation in striatum. Disruption of striatal D1-DA receptor activation decreases cortical excitability (Steiner and Kitai 2000; Yano *et al.* 2006; Gross and Marshall 2009). Additionally, cortical immediate-early gene induction by DA receptor agonists is blunted in rats with METH-induced neurotoxicity (Belcher *et al.* 2009). Furthermore, novelty-induced c-*fos* mRNA expression in striatopallidal neurons is dependent on corticostriatal transmission (Ferguson and Robinson 2004), sensitive to decreases in D1-DA receptor activation (Ferguson *et al.* 2003), and sensitive to blockade of NMDA receptors or ERK1/2 signaling (Ferguson *et al.* 2003; Ferguson and Robinson 2004). These data thus suggest a model, wherein loss of D1-DA receptor activation in striatum because of disrupted phasic signaling secondary to METH-induced monoamine toxicity may lead to decreased cortical excitability and, consequently, decreased gene expression in *ppe*-pos neurons.

An alternative explanation for the overall loss of novelty-induced *Arc* transcription is that chronic elevations in basal *Arc* mRNA expression suppress further transcriptional activation. Massed exposures to a spatial environment in a single day impair *Arc* transcription (Guzowski *et al.* 2006) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor activation can inhibit *Arc* transcription (Rao *et al.* 2006). Although there are DNA regulatory elements that repress *Arc* transcription (Pintchovski *et al.* 2009), what binds to those regulatory sites and the situations under which they are engaged are currently undefined. Therefore, the extent to which transcriptional repression of the *Arc* gene versus impaired activation of post-synaptic receptors (NMDA, D1) or intracellular signaling cascades contributes to the loss of evoked *Arc* expression in rats with METH-induced neurotoxicity remains to be determined.

The present results confirm prior observations (Daberkow *et al.* 2007, 2008) that normal, saline-pre-treated animals have more *ppe*-neg neurons than *ppe*-pos neurons with *Arc* mRNA in the cytoplasm. At present, the basis for this phenotypic difference in normal animals remains unknown, but it may be related to differences in *Arc* trafficking or cytoplasmic mRNA stability, given that *Arc* mRNA is a substrate for translation-dependent decay (Giorgi *et al.* 2007). The lack of transcriptional activation of *Arc* in rats with METH-induced neurotoxicity makes it somewhat difficult to discern whether DA contributes to this phenotypic difference; however, the present results suggest that the trafficking/stability of *Arc* mRNA in *ppe*-neg neurons in normal animals is dependent on DA signaling. First, despite the lack of novelty-induced transcriptional activation, METH-pre-treated, CC rats have more *ppe*-pos neurons with cytoplasmic *Arc* than do saline-pre-treated, CC rats, suggesting that METH-pre-treated rats still traffic basally transcribed *Arc* mRNA into the cytoplasm in *ppe*-pos neurons. Conversely, despite having high basal numbers of *ppe*-neg neurons with transcriptional activation of *Arc*, METH-pre-treated, 30-min rats do not have greater numbers of *ppe*-neg neurons with cytoplasmic *Arc* expression compared with saline-pre-treated, 30-min rats. Thus, the elevated basal transcription of *Arc* mRNA in *ppe*-neg neurons of METH-pre-treated rats does not translate into greater numbers of neurons with cytoplasmic *Arc*, suggesting some disruption of *Arc* trafficking/stability in *ppe*-neg neurons. Second, whereas there is a slight, but significant, increase in the numbers of *ppe*-pos neurons with cytoplasmic *Arc* mRNA in the METH-pre-treated, 30-min group relative to the numbers in the METH-pre-treated, CC group, there is no such increase in the numbers of *ppe*-neg neurons. Taken together, these observations suggest that METH-induced neurotoxicity is associated with impaired acute transcriptional activation of *Arc* in both subtypes of striatal efferent neurons, as well as impaired trafficking/stability specifically in *ppe*-neg neurons.

We presently report that a neurotoxic regimen of METH has significant, long-term impact on the regulation of transcriptional activation and subcellular expression of *Arc* mRNA. This METH-induced effect on *Arc* mRNA transcription and expression may thus impair Arc protein translation, with Arc protein synthesized within 60 min of neuronal stimulation (Lyford *et al.* 1995; Vazdarjanova *et al.* 2006; Baez *et al.* 2011). Importantly, this disrupted activity-dependent striatal *Arc* expression, and hypothesized impact on protein synthesis, may underlie previously reported changes in basal ganglia-mediated learning and memory processes in METH-pre-treated rats, including response-reversal (Daberkow *et al.* 2008; Pastuzyn *et al.* 2012), sequential motor (Chapman *et al.* 2001), and stimulus-

response versus action-outcome (Son *et al.* 2011) learning. Our present results therefore suggest *Arc* mRNA or the factors regulating it as potential novel therapeutic targets in the treatment of METH-induced cognitive impairments (Volkow *et al.* 2001; Kalechstein *et al.* 2003; Johanson *et al.* 2006), as Arc is a critical mediator underlying normal neuroplasticity processes (Chowdhury *et al.* 2006; Rial Verde *et al.* 2006). Future work will thus need to examine whether therapeutically restoring partial DA loss will resolve the molecular and cellular changes that occur, as well as improve cognitive functionality compromised with METH addiction and abuse.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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CHAPTER 5

DISCUSSION AND PERSPECTIVES

Summary of mRNA Regulation within Striatal Efferent Neurons

The basal ganglia critically mediate normal motor behaviors and action selection. The striatum is the key input nucleus of the basal ganglia, and within the striatum, two discrete populations of spiny efferent neurons exist that mediate normal control of motor behavior and action selection [1-3]. Thus, unraveling normal neuroplasticity processes within the striatum, as well as how neurodegeneration disrupts such signaling, is critical to advancing our understanding of basal ganglia function. Importantly, the immediate-early gene *Arc* provides a useful neuronal tool to examine such neuroplasticity within the striatum due to its key role in mediating normal LTP, LTD, and homeostatic plasticity processes [4]. Such understanding of *Arc* mRNA regulation, as well as general processes mediating mRNA regulation, in striatal efferent neurons will allow further identification of novel therapeutic targets for the treatment of basal ganglia-mediated neurological and neurodegenerative disorders.

We have previously demonstrated that following behavioral activation by exploration of a novel environment, *Arc* mRNA expression is robustly induced in phenotypically identified striatal efferent neurons of normal animals [5]. While equivalent numbers of striatonigral and striatopallidal neurons showed transcriptional activation of the *Arc* gene in response to this behavioral activation, 30 minutes after novel environment exposure, there

were more striatonigral neurons with *Arc* mRNA in the cytoplasm than striatopallidal neurons [5]. Differences in posttranscriptional mRNA processing within the nucleus, in cytoplasmic mRNA trafficking, or in translational regulation in striatal efferent neuron subpopulations conceivably could contribute to this phenotypic difference in the numbers of cells with cytoplasmic *Arc* mRNA. Importantly, *in vitro* studies demonstrate that *Arc* mRNA is regulated at the posttranscriptional level by the RNA-binding protein, eukaryotic initiation factor 4A3 (eIF4A3), and the exon-junction complex (EJC)[6,7]. Following the first round of protein translation, the EJC can target *Arc* mRNA for degradation through the process of translation dependent decay (TDD) [6,8,9,7]. Thus, neuron specific differences in eIF4A3 could have potentially contributed to more striatonigral than striatopallidal neurons having cytoplasmic *Arc* mRNA expression in normal animals.

Additionally, rats that had a partial monoamine depletion induced by a neurotoxic regimen of methamphetamine (METH) demonstrated significant blunting of the numbers of cells with cytoplasmic *Arc* mRNA expression relative to saline-pretreated rats following performance on a striatally mediated T-maze task [10]. These observations additionally suggested that METH-induced monoamine depletion substantially disrupted *Arc* mRNA signaling in response to striatally mediated learning, but whether such effects were due to alterations in *Arc* mRNA transcriptional activation or cytoplasmic mRNA localization was, at the time, unknown.

Altogether, these previous observations that striatal efferent neurons differentially regulate *Arc* mRNA cytoplasmic localization and that METH-induced neurotoxicity disrupted normal transcriptional activation and subcellular localization of *Arc* required in-depth investigation to further understand the mechanisms regulating such mRNA expression and cytoplasmic localization within striatal efferent neurons. Thus, the studies presented in

this dissertation began to determine the factors contributing to the differences in cytoplasmic *Arc* mRNA localization within striatal efferent neurons. Furthermore, these studies examined how such mRNA regulation could be disrupted in the context of partial monoamine loss induced by METH. Herein, we presently report evidence that striatal efferent neurons differentially regulate immediate-early genes (IEGs) essential to synaptic plasticity processes and that partial monoamine loss induced by METH disrupts such normal mRNA regulation.

First, we characterized the mRNA and protein expression of EJC factors in the dorsal striatum and hippocampus following both novel environment exploration and striatally mediated learning on a T-maze task (Chapter 2)[11]. Through those studies, we demonstrated that the expression of both eIF4A3 mRNA and protein increased within the dorsal striatum in response to behavioral activation by exploration of a novel environment. However, expression of another EJC core component, *Magoh*, did not show such activity-dependent mRNA expression, further uniquely implicating eIF4A3 in neuroplasticity processes. Additionally, following striatally mediated learning on a T-maze task, which is known to require the dorsomedial (DM) striatum [5,12,13], only expression of *eIF4A3* mRNA in the DM striatum significantly correlated to behavioral performance, in a manner very similar to *Arc* mRNA [10]. Importantly, it has previously been suggested that only the expression of *Arc* mRNA within the brain region required for a particular task correlates to behavioral performance on that task [14]. Thus, our observations point to a potential role for eIF4A3 in *Arc*-dependent learning and memory processes. Altogether, these studies provided the first characterization of the expression of this critical component of the EJC in the adult mammalian brain and demonstrated a potential role for eIF4A3 in *Arc*-dependent neuroplasticity processes in the dorsal striatum.

We then went on to examine the expression of other synaptic plasticity-associated mRNAs that share discrete transcriptional and dendritic trafficking characteristics with *Arc* mRNA (Chapter 3). Specifically, we selected the transcription factor, *zif268/egr-1*, which is an IEG activated by exposure to a novel environment with a time course similar to that of *Arc* mRNA [14]. However, unlike *Arc* mRNA, *zif268* mRNA is not trafficked to activated synapses for localized protein translation [15,16]. Additionally, we investigated the expression of the constitutively expressed, dendritically trafficked mRNA, *Map2* [17,18]. Most importantly for these studies, neither *zif268* nor *Map2* mRNAs are subject to cytoplasmic mRNA regulation through EJC-dependent TDD [6], thereby making both mRNAs extremely useful tools to examine the cytoplasmic localization of mRNAs in striatal efferent neurons. Intriguingly, we found that there also were phenotypic differences in the numbers of striatonigral vs. striatopallidal neurons with cytoplasmic expression of *zif268* mRNA following exploration of a novel environment. Furthermore, closer examination of the subcellular expression of *zif268* mRNA 30 minutes after novel environment exploration revealed that more striatopallidal than striatonigral neurons had nuclear retention of *zif268* mRNA, suggesting that mRNA trafficking out of the nucleus in striatopallidal neurons may be different than that in striatonigral neurons. Such differences in nucleocytoplasmic trafficking could account for the greater number of striatonigral neurons with IEG mRNAs in the cytoplasm. Clearly, further studies are necessary to assess how general these differences are and whether the phenotypic differences in nuclear and cytoplasmic mRNA distribution in striatal efferent neurons are linked.

In the context of METH-induced DA depletion, cytoplasmic localization of *zif268* was significantly disrupted, despite the fact that transcriptional activation was essentially unchanged relative to saline-pretreated rats. Such examination of the activity-induced

subcellular localization of *zif268* demonstrates that striatal efferent neuron subpopulations may, in fact, undergo differential mRNA regulation in response to neuronal activation and that DA may play a role in mediating nuclear mRNA export. Finally, when we examined the expression of constitutively expressed *Map2* mRNA, there were no significant differences between striatal efferent neuron subpopulations in subcellular mRNA localization and numbers of cells with cytoplasmic *Map2* mRNA. Such observations suggest that acute regulation of nuclear posttranscriptional processing or mRNA export for IEGs may underlie these cytoplasmic localization differences. Altogether, these studies demonstrate that differences in the distribution of *Arc* mRNA to the peri-nuclear cytoplasm in striatal efferent neuron subpopulations does not appear to be regulated by eIF4A3 and the EJC [6], but rather may arise as consequence of striatal efferent neuron-specific differences in nuclear posttranscriptional mRNA processing and/or mRNA export.

Finally, we examined the induction and cytoplasmic localization of *Arc* mRNA within striatal efferent neurons subsequent to partial monoamine depletion induced by METH so as to determine whether the previously reported METH-induced disruption of cytoplasmic *Arc* expression after performance on a striatally mediated T-maze [10] was due to impaired transcriptional activation or cytoplasmic localization of *Arc* mRNA (Chapter 4). In these studies, we found that long-term monoamine depletion induced by METH results in significant elevations in basal *Arc* mRNA levels within striatal efferent neurons, but also complete abrogation of transcriptional activation of *Arc* mRNA in response to novel environment exploration, in contrast to that seen in normal animals [19]. Additionally, activity-dependent localization of *Arc* mRNA to the cytoplasm was dysregulated, with striatonigral neurons showing considerable disruption of cytoplasmic *Arc* localization. Furthermore, we also demonstrated that the extent of DA depletion correlated with this

disruption of cytoplasmic *Arc* mRNA expression, in line with previous reports that partial DA depletion more significantly impairs striatonigral neuron function [20-22]. Given that the degree of DA depletion also correlates with the degree of disruption in phasic DA signaling [23] and that phasic DA signaling may selectively alter D1 DA receptor-mediated signaling [24-28], these findings suggest that DA activation of the D1 receptor may critically contribute to posttranscriptional mRNA processing in striatonigral efferent neurons. Importantly, given its critical role in synaptic plasticity underlying consolidation of learning and memory, disruption of *Arc* mRNA transcriptional activation and cytoplasmic localization may likely contribute to behavioral and cognitive deficits in humans with a history of METH abuse [29-32].

Role of mRNA Regulation in Diseases of the Basal Ganglia

Many basal ganglia-mediated pathologies are caused by aberrant synaptic plasticity due to disruptions in normal neuronal posttranscriptional processing, cytoplasmic mRNA stability, and dendritically localized protein translation. For example, mutations in the UPF3b protein in humans leads to disruption of nonsense-mediated mRNA decay (NMD) and cytoplasmic mRNA regulation and results in childhood-onset schizophrenia and autism spectrum disorders (ASDs [33]), as well as mental retardation with or without ASDs [34,35]. Interestingly, UPF3b, and more generally NMD, can be regulated by the micro-RNA, miR-128 [36], whose levels have also been shown to be deregulated in transgenic mouse models of basal ganglia pathologies, including Huntington's disease [37] and Parkinson's disease [38]. Altered translational regulation at activated synapses can also disrupt normal synaptic plasticity. For example, enhanced levels of protein synthesis due to a single-nucleotide polymorphism in the promoter of the cap-binding *eIF4E* gene in humans leads to ASDs [39-

41], and *eIF4E*-transgenic mice also exhibit autistic-like behaviors, including excessive repetitive and perseverative behaviors, and synaptic pathophysiology within striatum [42]. Finally, disorganized mRNA localization of plasticity-associated mRNAs can underlie pathologies of the basal ganglia. The RNA-binding protein Fragile X Mental Retardation Protein (FMRP) interacts with the brain-predominating nuclear export factor, NXF2 [43], and is essential to mRNA (including *Arc*) localization to activated synapses [44,45]. Dysfunction of FMRP is implicated in the ASD, Fragile X syndrome (FXS; [46-49]). Given that there is disruption of GABAergic neurotransmission and cortical excitability in FXS [50,51], dysfunction in FMRP-mediated protein trafficking may also disrupt striatal neuron function at both the pre- and postsynaptic levels through the deregulation of local protein synthesis essential to normal synapse formation and synaptic plasticity. Therefore, dysfunction of mRNA stability, localization, and dendritic protein synthesis can have profound effects on normal neuronal function. Thus, improving our understanding of normal mRNA regulatory processes in striatal projection neurons will allow for more comprehensive treatment strategies to ameliorate aberrant neuroplasticity processes in disorders of the basal ganglia.

The loss of dopamine neurons in the substantia nigra pars compacta coupled with decreased dopamine input to striatum leads to hallmark symptoms of Parkinson's disease (PD) [52]. Replacement of endogenous dopamine with L-DOPA is the most beneficial therapy currently available to PD patients [53]. However, the subsequent development of L-DOPA-induced dyskinesia is one of the most problematic side effects in the treatment of PD [54]. These debilitating dyskinesias likely result from aberrant synaptic plasticity at corticostriatal synapses [55,56]. In light of our present studies on *Arc* mRNA regulation within the partially dopamine-denervated striatum, it is interesting Arc protein is significantly

upregulated and correlates with the severity of L-DOPA-induced dyskinesia in a rat model of L-DOPA-induced dyskinesias [57]. Furthermore, chronic L-DOPA treatment in 6-OHDA-lesioned rats preferentially induces *Arc* mRNA expression within striatonigral neurons, which are also the neuron population more severely affected by partial monoamine loss induced by METH [19,22,20,10]. Thus, our present observations for disrupted *Arc* mRNA cytoplasmic localization following partial monoamine depletion (Chapter 4 [19]), as well as the fact that *Arc* mRNA and protein are upregulated in response to chronic L-DOPA, further support a model whereby cytoskeletal remodeling and aberrant synaptic plasticity within striatonigral neurons may lead to the development of dyskinesias during the course of PD treatment [58]. *Arc* mRNA and the factors and cellular processes regulating it are, therefore, potentially critical therapeutic targets in the amelioration of adverse effects associated with the pharmacological management of PD.

Lastly, the present studies highlight potential differences in striatal efferent neurons in the regulation of nuclear mRNA export of activity-regulated genes. Striatal efferent neurons express different dopamine receptors [59], with downstream cellular signaling mediated by the cellular response to dopamine [60,61]. Importantly, we now demonstrate that in normal animals, there are striatal efferent neuron-specific differences in cytoplasmic mRNA localization of the IEGs *Arc* (Chapter 4) and *zif268* (Chapter 3) mRNA. Furthermore, animals with METH-induced dopamine depletion have blunted activity-dependent cytoplasmic localization of *Arc* (Chapter 4) and *zif268* (Chapter 3) mRNA relative to normal animals. These results point to a key role for dopamine in contributing to such divergent numbers of striatal efferent neurons with cytoplasmic mRNA localization and highlight the possibility for cell-specific regulation of nuclear mRNA export. It is possible that striatal efferent neuron subpopulations do employ different regulatory mechanism for

nuclear mRNA processing, as several reports have recently suggested cell-specific regulation of factors essential to mRNA processing. First, specific nuclear TREX components, which are essential to normal nuclear mRNA processing and export [62,63], play component-specific roles in mouse embryonic development (THOC1 [63-66]), adipocyte differentiation (THOC5 [67]), or cancer development (THOC5 [68,69]). Second, both the proteins ALY/REF and the TREX component, THOC1, show tumor-specific expression differences, as well as variations in regional expression [69]. Lastly, TREX components are differentially regulated in the mouse testis and specifically regulate the expression of a subset of genes in this region [70]. Such observations are intriguing in light of our present observations, because the expression of *Arc* mRNA regulatory factors, BDNF [71] and NMD components [72,36], are also highly expressed in the testes, second only to expression within the mammalian brain, suggesting by analogy that there may also be neuron-specific roles for these factors in the regulation mRNAs, such as *Arc*. Thus, these recent studies suggest the possibility for tissue- and gene-specific regulatory roles for nuclear processing factors essential to nuclear mRNA export [63]. How these export factors are themselves expressed and regulated within striatal efferent neuron subpopulations remains to be determined. Further understanding the neuron-specific regulation of IEG nuclear export within striatal efferent neurons would offer essential understanding of normal basal ganglia-dependent functions, as well as identify novel therapeutic targets in the treatment of basal ganglia-mediated pathologies.

Future Directions

Herein, we demonstrate that striatal efferent neuron subpopulations appear to differentially regulate nuclear posttranscriptional mRNA processing or nuclear export of the

activity-regulated mRNAs *Arc* and *zif268*, but not constitutively expressed *Map2*, in response to novel environment exploration. Unlike *Arc, zif268* is not a candidate for cytoplasmic translation-dependent mRNA decay mediated by eIF4A3 and the EJC [6]. Thus, EJC-dependent mRNA decay likely does not contribute to the observed differences in cytoplasmic *Arc* mRNA expression within striatopallidal vs. striatonigral neurons [5,19]. Despite the fact that eIF4A3 shares a similar expression profile and activation kinetics with *Arc* mRNA in response to behavioral activation and striatally-mediated learning [11], our observations that *zif268* also shows differential cytoplasmic distribution within striatal efferent neurons suggests two potential alternative mechanisms contributing to the phenotypic differences in cytoplasmic mRNA expression. Thus, future studies must carefully examine these two potential mechanisms so as to definitively determine how cytoplasmic expression differences arise within striatal efferent neuron subpopulations.

The first potential mechanism contributing to the observed differences in numbers of cells with *Arc* and *zif268* in the cytoplasm could be a spatially restricted mechanism regulating mRNA cytoplasmic localization, such as nuclear posttranscriptional mRNA processing or nuclear export differences. Distribution of mRNA-protein (mRNP) complexes from the nucleus to the cytoplasm requires complex intracellular signaling and could thus be a critical processing point whereby neuronal mRNA transport is actively regulated [73,74]. Yet how nuclear export proceeds in neurons in general, and striatal efferent neurons in particular, requires further scrutiny. Future investigations into the regulatory processes mediating differences in nucleocytoplasmic mRNA localization within striatal efferent neurons are thus necessary. As described in Chapter 3, protein kinase A (PKA) has been shown to contribute to the nucleocytoplasmic export of proteins *in vitro* [75,76], but whether

this kinase also plays a potential role in the regulation of mRNA export in striatal efferent neurons remains to be determined.

Future studies must thus specifically examine how pharmacological manipulation of PKA *in vivo* alters the neuron-specific cytoplasmic localization expression of mRNAs, such as *Arc* and *zif268*. For example, preceding novel environment exploration with systemic administration of a D1-type DA receptor antagonist, such as SCH23390, would selectively inhibit activation of AC activity and thus production of PKA. Such D1-type DA receptor selective manipulations could thus potentially result in diminished numbers of striatonigral neurons with *Arc* or *zif268* in the neuronal cytoplasm 30 minutes after novel environment exploration. Conversely, preceding novel environment exploration with systemic administration of A2A-receptor antagonists, such as istradefylline or preladenant, would selectively activate PKA only within striatopallidal neurons [77,78]. This alternative pharmacological treatment could then potentially augment the numbers of striatopallidal neurons with cytoplasmic IEG expression 30 minutes after novel environment exploration. These neuron-specific pharmacological manipulations would thus suggest that the key differences in nuclear mRNA export within striatal efferent neuron subpopulations likely arise due to differential activation of PKA mediated by neuron-specific DA receptors.

The second alternative is that striatal efferent neurons have neuron-specific differences in cytoplasmic mRNA degradation that do not depend on the EJC, thus potentially contributing to the observed phenotypic differences in numbers of cells with cytoplasmic *Arc* and *zif268* mRNA. While *Arc* mRNA is a candidate for NMD initiated by continued presence of EJCs within the 3'UTR following the first round of protein translation [6,79], the IEG *zif268* is not a candidate for such mRNA regulation [6]. Additionally, while the core EJC component eIF4A3 showed an expression profile within

the adult rodent brain very much like *Arc* mRNA (Chapter 2 [11]), the fact remains that mRNA degradation can proceed through multiple mechanisms, which are only beginning to be fully understood [72,80,81]. Thus, while *zif268* may not be subject to *Arc*-like, EJC-dependent NMD, it is possible that alternative mechanisms of mRNA decay mediated by EJC-independent pathways (i.e., UPF2/RNPS1-mediated NMD [81] or Staufen1-mediated mRNA decay (SMD [80])) could also potentially contribute to the observed phenotypic differences in numbers of striatal efferent neurons with cytoplasmic mRNA. At this point, it is unknown whether *Arc* or *zif268* are candidates for these alternative branches of mRNA decay, although *Arc* does associate with Staufen1 [44] and could thus also be regulated by this pathway. Interestingly, both the EJC-dependent and independent branches of NMD (i.e., UPF2/RNPS1-activated), as well as SMD, all initiate mRNA degradation through a convergent mechanism: phosphorylation of UPF1 [72,82,80,83,81]. Preliminary examination of UPF1 phosphorylation within dorsomedial striatum following spatial exploration of a novel environment revealed a significant time-dependent increase in total field area with phosphorylated-UPF1 signal (Barker-Haliski and Keefe, unpublished). Thus, while UPF1 phosphorylation may show activity-dependent increases in the adult mammalian striatum, the significantly delayed profile of phospho-UPF1 does not support the more rapidly observed differences in numbers of cells with cytoplasmic *Arc* and *zif268*. Additionally, there are more striatopallidal neurons with *zif268* in the nucleus 30 minutes after novel environment exploration (Chapter 3), thereby making differences in cytoplasmic mRNA degradation a less-likely factor contributing to neuron-specific differences in numbers of cells with IEGs in the cytoplasm. Thus, future studies must more closely examine discrete markers of mRNA degradation pathways within striatal efferent neurons so as definitively

determine whether nuclear mRNA export or cytoplasmic mRNA decay underlie neuron-specific differences in cytoplasmic mRNA.

While our preliminary analysis examined regional changes in striatal UPF1 phosphorylation, it is possible that mRNA decay may still be differentially activated within striatal efferent neuron subpopulations, but that this difference cannot be readily identified by such global analysis of phosphorylated-UPF1. Such neuron-specific differential activation could thus also contribute to phenotypic differences in numbers of cells with mRNA in the cytoplasm. Future studies must thus examine the phenotype-specific phosphorylation state of UPF1 in response to neuronal stimulation paradigms, so as to clearly define whether mRNA decay is being differentially activated within striatal efferent neuron subpopulations. Furthermore, targeted pharmacological manipulation of mRNA decay components within striatal efferent neuron subpopulations could specifically tease apart the specific components contributing to such differences in cytoplasmic mRNA availability. For example, the general translation inhibitor cycloheximide (CHX) also specifically blocks NMD [84,85] by selectively disrupting the transient association of UPF1 with the EJC [82,86,87]. Although CHX still allows UPF1 phosphorylation, it stabilizes the association of UPF1 with the UPF3b-EJC-mRNA complex [88], thereby disrupting recruitment of the decay factors Dcp1a, Xrn1, and Rrp4 [82,86] and thus mRNA degradation. Conversely, the natural product Pateamine A (specifically, its active metabolite, *desmethyl,desamino*-Pateamine A (DMDA-PatA)) blocks EJC-dependent NMD by selectively trapping eIF4A3 in an unstable conformation, thereby disrupting the interaction of eIF4A3 with UPF1 and selectively inhibiting UPF1 phosphorylation necessary to initiate mRNA degradation [88,89]. Thus, these two inhibitors of NMD exert their effects by targeting different aspects of NMD activation, thereby providing useful tools to selectively examine one pathway of mRNA

decay within activated neurons. Furthermore, cytoplasmic expression of *zif268* mRNA within both neuronal phenotypes would likely be immune to such pharmacological manipulations *in vivo,* whereas *Arc* mRNA, which is subject to EJC-dependent NMD [6], should be disrupted by both CHX and DMDA-PatA treatment. However, if such *in vivo* studies on *Arc* and *zif268* show no change in numbers of cells with either IEG in the cytoplasm, it would further indicate that NMD does not contribute to the phenotypic expression differences within striatal efferent neuron subpopulations. Such studies using targeted pharmacological approaches could thus further define whether NMD is, in fact, playing a role in the phenotype-specific differences in numbers of cells with *Arc* or *zif268* in the cytoplasm.

Furthermore, using co-cultured primary striatal and cortical neurons from transgenic mice that selectively express reporter proteins for D1- or D2-type DA receptors [90], would allow for *in vitro* examination of NMD activation in isolated striatal neuron subtypes. Application of pharmacological compounds that target NMD, such as CHX or DMDA-PatA, prior to stimulation of D1- or D2-containing neurons could selectively define *in vitro* whether EJC-dependent NMD might differentially regulate IEG mRNA cytoplasmic expression. Should these studies show no neuron-specific changes in numbers of cells with cytoplasmic IEGs, it would further indicates that either an alternative branch of mRNA decay (i.e., SMD) or phenotypic differences in nuclear export are mediating such cytoplasmic expression differences. Thus, using isolated striatal neuron subtypes would more clearly define whether mRNA degradation is mediating changes in numbers of cells with cytoplasmic IEG expression, and if so, clarify the specific mRNA decay process involved.

Altogether, such in-depth investigations into the neuron-specific subcellular regulation of mRNAs examining either nuclear mRNA export processes or mRNA decay

pathways will more fully define the basis for neuron-specific differences in the numbers of striatal neurons with cytoplasmic IEG expression. Examination of these processes under both normal conditions and in the context of neurological and neurodegenerative disorders would offer a yet unexplored level of differential cellular regulation underlying neuroplasticity processes within striatal efferent neuron subpopulations. Such studies would thus provide innovative direction in developing novel therapeutic treatments for basal ganglia-mediated pathologies.

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